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Biotechnological production of fucosylated human milk oligosaccharides (HMO) and core structures thereof

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Kurzfassung Die biotechnologische Synthese von humanen Milch-Oligosacchariden (HMO) stellt eine vielversprechende Alternative zu chemischen Synthesen und der Isolierung von HMO dar.
Vor allem eine effiziente Produktion von fucosylierten HMO ist von außerordentlicher Bedeutung, da diese HMO prebiotische oder antigenische Wirkungen zeigen, die das Wachstum eines Kleinkinds unterstützen, was sie zu geeigneten Inhaltsstoffen für Säuglingsfertignahrung macht.
Die Entwicklung nährreicher Säuglingsfertignahrung ist essentiell, da es Frauen gibt, die effiziente Mengen an fucosylierten HMO - insbesondere 2`-Fukosyllactose und andere 2`-fukosylierte HMO- während der Laktation nicht produzieren können.
In solchen Fällen ist eine gesundheitsfördernde Alternative zu natürlicher Muttermilch von Bedeutung.
Diese Diplomarbeit befasst sich mit biotechnologischen Synthesen verschiedener fucosylierter HMO beziehungsweise Kernstrukturen dieser, welche die Entwicklung und Gesundheit von Babys fördern.
Sie gibt einen Überblick über verschiedene Trends in der Biotechnologie bezüglich Produktion at large scale, der Verwendung bestimmter fucosylierender Enzyme sowie biotechnologischer Systeme basierend auf unterschiedlichen Engineering Strategien und vergleicht sie hinsichtlich ihrer Effizienz.

Schlüsselwörter Fucosylierte humane Milch-Oligosaccharide (HMO) – Prebiotische und antigenische Wirkungen – Säuglingsfertignahrung – 2`-Fucosyllaktose – Biotechnologische Synthese fucosylierter HMO

Abstract The biotechnological synthesis of human milk oligosaccharides (HMO) represents a promising alternative to chemical syntheses and isolation of HMO.
In particular, the efficient production of fucosylated HMO is of significant importance since these HMO show prebiotic or antigenic effects supporting growth of an infant which makes them convenient ingredients of infant formula.
The development of nutritious infant formula is essential since there are women that are not able to produce efficient amounts of HMO – mainly 2`-fucosyllactose and other 2`-fucosylated HMO-during lactation.
Thus, supplying their babies with a health- promoting alternative to natural mother milk is of importance.
This diploma thesis deals with biotechnological syntheses of various fucosylated HMO respectively core structures thereof that support development and health of babies.
It gives an overview of different trends in biotechnology concerning production at large scale, the use of certain fucosylating enzymes as well as biotechnological systems based on various engineering strategies and compares them with regard to their efficiency.

Keywords Fucosylated human milk oligosaccharides (HMO) – Prebiotic and antigenic effects – Infant formula – 2`-fucosyllactose – Biotechnological synthesis of fucosylated HMO

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Biotechnological production of fucosylated human milk oligosaccharides (HMO) and core structures thereof

Chapter I

Review Biotechnological production of fucosylated human milk oligosaccharides (HMO) and core structures thereof

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Abstract The biotechnological synthesis of human milk oligosaccharides (HMO) represents a promising alternative to chemical syntheses and isolation of HMO.
Especially the production of fucosylated HMO is of importance since these HMO are used in infant formula because of their prebiotic or antigenic effects that support growth of an infant. The development of nutritious infant formula is essential because there are women that do not want to breastfeed babies or women that are Lewis blood group negative and not able to produce appropriate amounts of HMO during lactation.
This review gives an overview of biotechnological synthesis pathways of different fucosylated HMO respectively core structures thereof that support the health of babies. Trends in biotechnology will be suggested concerning the production of fucosylated HMO at large scale with focus on various donor substrates, acceptors and fucosylating enzymes.
In general two main strategies in order to synthesise fucosylated HMO will be introduced: On the one hand the in vivo synthesis by engineered cell expression systems harbouring genes for fucosyltransferase, NADPH cofactor recycling or GDP-fucose formation within the cell. On the other hand the in vitro synthesis by various fucosidases, fucosynthases and fucosyltransferases.
These biotechnological in vitro and in vivo production systems adapted for the synthesis of certain fucosylated HMO will be compared concerning yield and amount of end product. Particularly alternative production systems of 2`-fucosyllactose will be described and contrasted in this review because it is known that mainly 2`-fucosyllactose is responsible for protecting infants against certain infections that are caused by microbial pathogens.

Especially approaches with metabolically engineered *Escherichia coli* strains expressing fucosyltransferase FucT2 from *Helicobacter pylori* will be accurately described because they turned out to be convenient for producing 2`-fucosyllactose in double-digit gram quantities.

Keywords Fucosylated human milk oligosaccharides (HMO) – Prebiotic and antibacterial effects – Infant formula – Biotechnological synthesis of fucosylated HMO

Introduction

Human milk represents the predominant diet of newborn infants (Chichlowski et al. 2011) and consists of the 4 substantials lactose, which is the most frequent component, lipids, especially triglycerides, proteins such as caseins and complex lactose based oligosaccharides.
These oligosaccharides are varying between 10 and 20 g/L milk (Kunz et al. 2000; Montreuil et al. 1960) and are quantitatively the third largest and most diverse component of breast milk after lactose and lipids.
So far, more than 130 unique structures of human milk oligosaccharides (HMO) were characterized (Kobata 2010; Urashima et al. 2011) and experimental evidence for the existence of a minimum of 100 additional species has been reported (Stahl et al. 1994; Wu et al. 2010).
Especially fucosylated HMO are of importance in supporting health of infants because they protect them against infections since in the first year of life a stable population of microflora is established in the human gut as a complex ecology that includes more than 400 interdependent species of bacteria inclusively microbial pathogens (Newburg et al. 2004).
HMO function as soluble ligand analogs to bacterial pathogens through their glycoconjugate structure respectively epitops so that they can adhere to them and block pathogen adhesion to the cell surface whereby the risk of an infection or a disease strongly depends on the amount of HMO within mother milk (Han et al. 2012).
For instance diarrhea in breast-fed infants by *Campylobacteror jejuni* is lowered by higher concentrations of oligosaccharides that contain H type 2 epitopes (Bode 2006) or 2`-fucosyllactose (Morrow et al. 2011) in mother milk and a resistance against Stable Toxin producing *Escherichia coli* is thought to be associated with the presence of α1,2- linked fucosylated oligosaccharides (Harris et al. 2005).
In general it can be said that humans that are blood group Lewis negative and show an absence of 2`-fucosylated HMO as a result are exposed to an increased risk for infections by

Escherichia coli (Newburg et al. 2004; Harris et al. 2005), *Campylobacter jejuni* (Morrow et al. 2004; Ruiz-Palacios et al. 2003; Bode 2006) or *Vibrio cholerae* (Ruiz-Palacios et al. 2003) as well as for diseases such as diarrhea (Harris et al. 2005), necrotizing enterocolitis (Morrow et al. 2011) or Crohn's disease (McGovern et al. 2010).

Beside their role in inhibiting the colonization and growth of pathogens competitively in order to protect the cell surface (Chichlowski et al. 2011) of the new borne, HMO may be further responsible for a prebiotic effect which mainly enhances colonization of bacterium *Bifidobacterium bifidum* (Newburg 2000; Charturverdi et al. 2001).

Especially fucosylated HMO are associated to support the development of a new borne. These HMO can be fucosylated directly at a lactose core molecule or at a N-acetylglucosamine residue.

So far, just two carbohydrate positions of lactose can be fucosylated in humans although a lactose molecule principally contains eight possible carbohydrate positions with hydroxyl groups as can be seen in figure 1a.

Beside a fucosylation at carbohydrate position C2 of the galactose residue, a further fucosylation at carbohydrate position C3 of the glucose residue within the lactose molecule occurs in humans, resulting in the formation of the two fucosyllactoses called 2`-fucosyllactose and 3`-fucosyllactose (Chaturvedi et al. 2001).

Both fucosyllactoses and their precursor lactose are illustrated in figures 1a-1c:

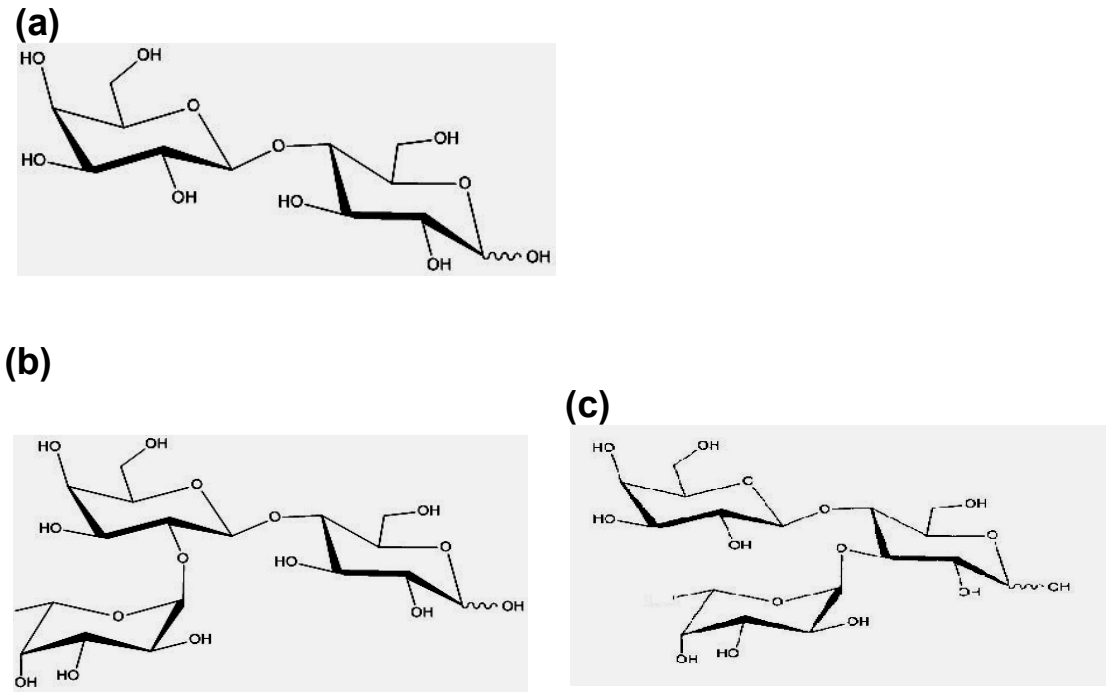


Fig. 1 Structures of lactose (a), 2`-fucosyllactose (b) and 3`-fucosyllactose (c).

Structures are drawn in ChemDraw Pro 13.0

With the exception of fucosyllactoses that are build up by 1-4 linked glucose and galactose residues in the form of lactose, nearly all examined fucosylated HMO that appeal as antigens or prebiotic stimuli for bacteria such as *Bifidobacterium bifidum* contain N-acetylglucosamine units with α1,3-glycosidic or α1,4-glycosidic bound fucoses as can be seen in oligosaccharide structures of table 1 (Castanys-Muñoz et al. 2013).

Two N-acetylglucosamine units are known to be involved in human fucosylation processes, namely the dominant type 1 chains Gal(β1-3)GlcNAc and type 2 units Gal(β1-4)GlcNAc that contain lactose and are called N-lactosamines (LacNAc) as a reason.

Human milk contains 4 mayor α1,2-fucosylated neutral oligosaccharides (Chaturvedi et al. 2001; Kobata 2010) fucosylated on the lactose core called lacto-N-fucopentaose I (LNFP I), lacto-N-difucohexaose I (LNDFH I), difucosyllactose (dFL) and finally 2`-fucosyllactose (2`FL) which represents the main component of fucosylated human milk oligosaccharides beside LNFP I (Prieto 2012).

In addition to fucosyllactoses 2`-fucosyllactose and 3`-fucosyllactose that are fucosylated at the lactose core there are also three mayor neutral oligosaccharides found in human mother milk with α1,3-and α1,4-fucosyl linkages at a N-acetylglucosamine residue.

These oligosaccharides are lacto-N-fucopentaose II (LNFP II) and lacto-N-difucohexaose II (LNDFH II) that are α1,4 fucosylated at N-acetylglucosamine as well as lacto-N-fucopentaose III (LNFP III) which is α1,3-fucosylated at N-acetylglucosamine (Charturverdi et al. 2011; Kobata 2010).

In table 1 important fucosylated neutral HMO and lactose from human mother milk are summarised:

Table 1 Structures of important fucosylated neutral HMO and lactose

Name	Abbreviation[a]	Structure[b]
Lactose	Lac	Galβ1-4-Glc
2`-fucosyllactose	2`-FL	Fucα1-2Galβ1 -4-Glc
3`-fucosyllactose	3`-FL	Galβ1-4(Fucα1-3)Glc
Difucosyllactose	DFL	Fucα1-2Galβ1 -4(Fucα1-3)Glc
Lacto-N-fucopentaose I	LNFP I	Fucα1-2Galβ1 -3GlcNAcβ1-3Galβ1-4Glc
Lacto-N-fucopentaose II	LNFP II	Galβ1 -3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc
Lacto-N-fucopentaose III	LNFP III	Galβ1 -4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc
Lacto-N-difucohexaose I	LNDFH I	Fucα1-2Galβ1 -3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc
Lacto-N-difucohexaose II	LNDFH II	Galβ1 -3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc

[a] Abbreviations were cited in Castanys Muñoz et al. (2013)

[b] Structures are adapted from Castanys-Muñoz et al. (2013), Kobata et al. (2010) and Kunz et al. (2000)

Because of the fact that fucosylated HMO play important roles in the development of the new borne an awakening interest in the biotechnological or chemical production as well as isolation of these HMO from natural resources emerged in the last years with focus on developing appropriate infant formulas.

Principally the isolation of fucosylated HMO from mammals such as cows would be a readily accessible method compared to biotechnological in vivo or in vitro processes.

Whereas in vivo approaches are based on time intensive engineering strategies or expensive starting materials (Maertens et al. 2013), in vitro processes often depend on enzymes that show disadvantages concerning abundance, specificity or product yield concomitant with cost intensive efforts (Lee et al. 2009).

However, important fucosylated HMO such as lacto-N-fucopentaose I-III or lacto-N-difucohexaose could not be observed in bovine milk (Kunz et al. 1999; Montreuil and Mullet 1960) which reveals that this method is rather inappropriate to yield high amounts of fucosylated HMO for infant formula.

Principally there exist also the oportunity to go back to chemical syntheses that are based on methods like automated solid-phase oligosaccharide synthesis (Plante et al. 2001) which can be applied to produce complex oligosaccharides such as the Lewis X or Lewis Y oligosaccharides within one day.

The oligosaccharides synthesized through this method are linked to insoluble material like beads or resins that allow the fast separation of reaction products from excess reagents, soluble reaction by-products and solvents (Seeberger and Werz 2005).

Further on a relative new approach of a crystalline intermediate technology was developed based on the protection and deprotection of saccharides so that synthesis of 2′-O-fucosyllactose is possible (Dékany et al. 2010).

Although the development of chemical synthesis probably leads to improvements such as higher purification, velocity or amounts of HMO, several hurdles for the industry-scale synthesis of HMO such as low overall yields, the use of toxic reagents that are not suitable for food products such as infant formula or low stereo-selectivity are still known (Han et al. 2012).

As a result the chemical synthesis of oligosaccharides often represents an inefficient but elaborate multi-step process- especially for stereo-selective synthesis- based on building blocks that must be selectively protected, then coupled and finally deprotected to yield a modification at a certain position (Palcic 1999).

Therefore biotechnological methods based on in vivo or in vitro systems represent a promising alternative to chemical syntheses in order to synthesize fucosylated HMO at large scale and support infant formula- research or clinical development.

An economic analysis puts the cost of oligosaccharide synthesis through in vivo syntheses at a price range from $30–50$/g product (Jianbo et al. 2003; Ruffing and Chen 2006) which can be regarded as an achievement of metabolic engineering and the basis that some oligosaccharides such as 2`-fucosyllactose and Lewis X oligosaccharides are now available in gram quantities (Baumgärtner et al. 2013;Koizumi et al. 2000) and at realistic costs (Ruffing and Chen 2006).

Enzymatic in vitro synthesis of fucosylated human milk oligosaccharides (HMO)

Various α-L-fucosidases, α-L-fucosynthases and α-L- fucosyltransferases were identified to be able to synthesize fucosylated HMO in vitro but each of them showed certain disadvantages concerning abundance, specificity or product yield concomitant with cost intensive efforts (Lee et al. 2009).

One advantage of fucosidases compared to fucosyltransferases is the high solubility and broader specificity of donor and acceptor substrates (Wymer and Toone 2000) whereas α-1,2-fucosyltransferases are strictly dependent on availabilities of expensive sugar nucleotide type donors (Lee et al. 2009).

On the other hand α-L-fucosidases show an additional hydrolysing activity beside a transfucosylating activity (Wada et al. 2008) which stands for a handicap in the synthesis of fucosylated HMO.

The main problem concerning the use of these glycosidases is mostly based on the fact that the desired product of the reaction is coincident a substrate for the enzyme because of the mentioned hydrolysing activity and therefore product yields are generally low compared to the synthesis of HMO by fucosyltransferases (Wada et al. 2008).

Therefore reaction conditions including acceptor sugar concentrations, the use of donor sugars with appropriate leaving groups or the reduction of water concentration through co-solvent addition (Rakic et al. 2009) and often directed evolution as was important for retaining α-fucosidase from *Thermotoga maritima* (Osanjo et al. 2007) have to be optimized to reduce hydrolysis and to increase fucosylation by α-fucosidases.

In the case of the retaining α-fucosidase from *Thermotoga maritima* mutations of gene *TM0306* were introduced through random mutagenesis by Taq PCR (Error Prone PCR) which led to mutations T264A, L322P, Y237H and Y267F responsible for improving transfucosylating activity.

Yields of the fucosylated product pNP Fuc(1-2)Gal from a reaction containing α-fucosidase of *Thermotoga maritima*, donor pNP-αFuc and acceptor pNP-βGal could be increased 4 till 6 times compared to the wild type enzyme showing a low yield of only 7%.

Further on recombination processes between clones harbouring the described mutation positions improved transfucosylating activity additionally so that product yields higher than 60% could be reached whereby the transglycosylation to hydrolysis ratio of α-fucosidase from

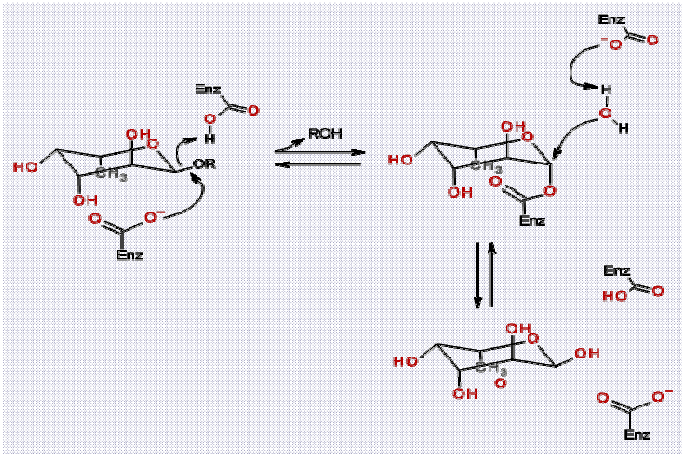
Thermotoga maritima could be increased significantly by up to 30 times according to the wild type enzyme (Osanjo et al. 2007).
Sometimes also natural α-fucosidases without further genetic modifications show a strong activity concerning fucosylation as well as low hydrolysing activity as could be seen on the inverting α-fucosidase AlfC from *Lactobacillus casei* which was able to synthesise fucosyl-α-1,6-N-acetylglucosamine with N-acetylglucosamine (GlcNAc) as acceptor and pNP-α -L-fucopyranoside (pNP-fuc) as donor at a high product yield of 56%, the highest one described for a wild-type fucosidase.
According to Rodríguez-Díaz et al. (2013) the efficient formation of fucosyl-α-1,6-N-acetylglucosamine means that this disaccharide may not be the natural substrate of AlfC.
Principally it may be also possible to use fucosyltransferases for the in vitro synthesis of fucosylated HMO but they are often membrane-bound and thus difficult to isolate and purify (Dékany et al. 2012).
Therefore fucosyltransferases may be more convenient for in vivo systems where they act as whole cell catalysts because optimal conditions do exist within the cell and sugar nucleotide type donors can be efficiently regenerated (Lee et al. 2012; Lee et al. 2013).
There is also the opportunity to go back to α-L-fucosynthases instead of α-L-fucosidases and α-L-fucosyltransferases to synthesise HMO in vitro.
Two strategies have been suggested to construct a glycosynthase from an inverting glycosidase so that hydrolysis is inhibited and formation of HMO products by transglycosylation achievable (Wada et al. 2008).
The first one is based on a replacement of a general base residue with a non-catalytic residue, the second on a replacement of a residue holding a non-polar residue with a catalytic water molecule as was shown in a study by Wada et al. (2008).
A so called 1,2-α-L- fucosynthase could be developed from an inverting 1,2-α-L-fucosidase (AfcA) from *Bifidobacterium bifidum* by Wada et al. (2008) so that hydrolysing activity could be reduced but transfucosylating activity increased.
This enzyme requires an inexpensive β- fucosyl fluoride as the donor substrate in order to synthesise the desired α1,2-fucosylated oligosaccharides.
One main problem of this donor substrate is its missing stability in aqueous solution which is often needed for enzymatic synthesis whereas fucosidases can use more stable and commercially available donor substrates instead.
Beside their dependence on unstable β-glycosyl fluoride donors a further drawback of using fucosynthases is their poor commercial availability (Wada et al. 2008).

However, the study by Wada et al. (2008) showed that only a low maximum product yield of 2`-fucosyllactose of 6 % could be obtained by the use of a fucosynthase.

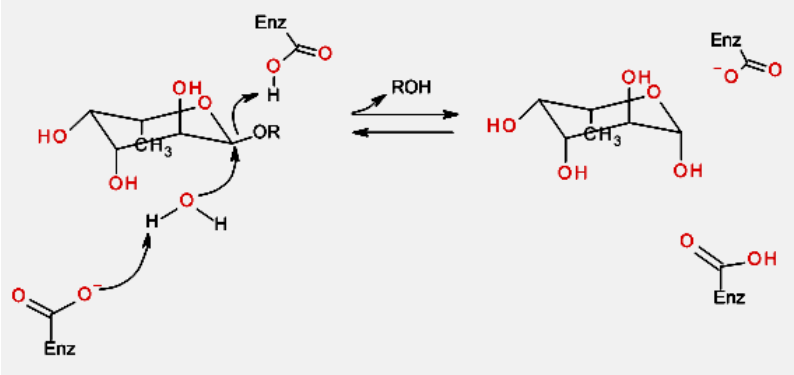
This fucosynthase was developed from the inverting 1,2-α-L-Fucosidase AfcA of *Bifidobacterium bifidum* by the replacement of an asparagine aspartic acid to a glycin residue. According to Wada et al. (2008) this modification was at least responsible for inhibiting the activation of an asparagine residue acting as a base whereby hydrolysis of 2`-fucosyllactose was avoided.

The typical hydrolytic reactions performed by an inverting and a retaining fucosidase as well as the catalytic mechanism of fucosylation by a fucosynthase that is developed from an inverting fucosidase are explained in figure 2:

(a) Retaining fucosidase



(b) Inverting fucosidase



(c) Fucosynthase

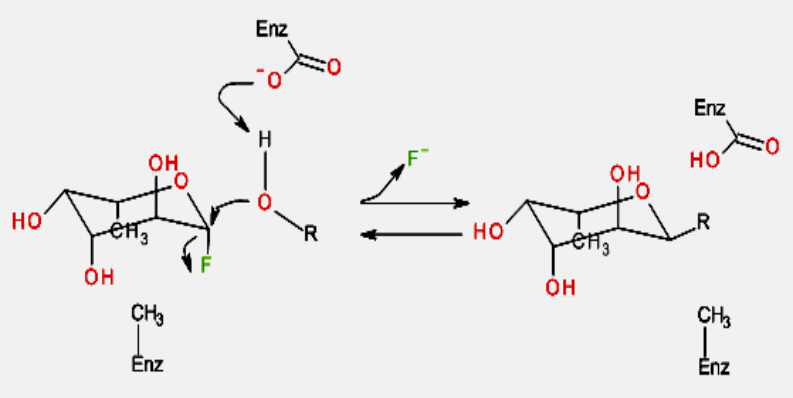


Fig. 2 Catalytic mechanism by retaining (a) and inverting fucosidase (b) as well as fucosynthase (c) drawn in Accelrys Draw 4.1. The catalytic mechanism by retaining (a) fucosidase is based on a double displacement process within the active centre in which a nucleophilic residue first attacks the anomeric center and a fucosyl-enzyme intermediate is achieved when a leaving group is protonated through an acid. The deprotonated acid functions as a base and thereby activates an incoming water molecule which leads to the hydrolysis of the fucosyl-enzyme intermediate and to a formation of a product with the same anomeric configuration as the substrate. If suitable acceptors enter the catalytic site instead of a water molecule, then transfucosylation is possible and the desired fucosylated product can be synthesized (Wada et al. 2008). The catalytic mechanism by inverting fucosidase (b) is based on a single displacement mechanism including a water molecule activated by a certain base that attacks the anomeric centre and a concomitant protonation of a fucosyl oxygen by a general acid. Thereby one carboxylic acid residue acts as general base and the other as general acid catalyst in order to obtain a bond cleavage and formation of a product with an inverted anomeric configuration (Wada et al. 2008). Inverting fucosynthases (c) catalyze the synthesis of glycoconjugates when a fucosyl fluoride donor of opposite configuration to the natural substrate is available. The fucosyl–enzyme intermediate is mimicked by the donor fucosyl fluoride and its sugar moiety transferred to the acceptor sugar. The hydrolysis of the glycoside (end product) is avoided through inactivation of a base function (Wada et al. 2008)

To sum up, the biotechnological enzymatic in vitro synthesis of various fucosylated HMO is still possible by retaining and inverting α-L-fucosidases, α-L-fucosyltransferases or α-L-fucosynthases.

These enzymes can be either used in their natural occurring structure or are genetically engineered to increase transfucosylating activity as could be demonstrated on the mentioned conversion of the inverting 1,2- α -L-fucosidase AfcA of *Bifidobacterium bifidum* into an 1,2-α-L-Fucosynthase (Wada et al. 2008) or the construction of fusion protein α1,2-fucosyltransferase WbsJ of *Escherichia coli* O128:B12 (Li et al. 2008).

In addition to in vitro approaches with one type of enzyme Nilsson et al. (1993) were able to demonstrate that the production of α-L-fucosyl-N-acetyllactosamines is possible by a combination of glycosidases and α-L-fucosyltransferase.

In table 2 biotechnological in vitro syntheses of various important fucosylated HMO or core structures thereof are summarized and compared concerning yield and amount of end product:

Table 2 Biotechnological in vitro production of different fucosylated HMO and core structures

HMO product	*Amount of enzyme (mg) respectively enzyme activity (U)*	*Concentration of donor substrate (mM)* [a]	*Concentration of acceptor substrate (mM)* [b]	*Amount of HMO (mg)*	*Final volume (mL)*	*Product yield (%)*	*Reference*
2`-FL	*Bifidobacterium bifidum* α -L-fucosynthase (0,07 mg)	β-l-fucosyl fluoride (10 mM)	Lactose (30 mM)	3	5	6	Wada et al. (2008)
2`-FL including structure	*Escherichia coli* α -L-fucosyltransferase (2 mg)	GDP-fucose (10 mM)	Gal-β1,4Glc-β-N3 (15 mM)	5,2	2	78	Li et al. (2008)
2`-FL including structure	Porcine liver α -L-fucosidase (0,45U)	Fucα-*p*NP (27,3 mM)	LacNAc (347,8 mM)	6,8	9	5	Murata et al. (1999)
3`-fucosyllactose including structure	*Alcaligenes* sp. α -L-fucosidase (0,6 U)	Fucα-*p*NP (10,5 mM)	LacNAc (260,9 mM)	60,8	20	54	Murata et al. (1999)
3`-fucosyllactose	*Alcaligenes* sp. α -L-fucosidase (0,6 U)	Fucα-*p*NP (10,5 mM)	LacNAc (182,5 mM)	35,2	20	34	Murata et al. (1999)
3`-fucosyllactose including structure	α -L-fucosidase (no enzyme activity and host strain listed)	No data available	No data available	No data available	No data available	42	Baer et al. (1980)

3`-fucosyllactose including structure	α -L-fucosidase (no enzyme activity and host strain listed)	No data available	No data available	No data available	No data available	67	Baer et al. (1980)
LNFP I and LNDFP I structure	Combination of Glucosaminidase (70 U), galactosidase (2 U) and α1-2 fucosyltransferase (0,04 U)	GDP-Fucose (22,6 mM)	Gal(β1-3)GlcNAc(β)-OMe (107,9 mM)	No data available	0,7	No data available	Nilsson et al. (1993)
LNFP III structure	Combination of glucosaminidase (70 U), galactosidase (2 U) and α1-3 fucosyltransferase (0,04 U)	GDP-Fucose (22,6 mM)	Gal(β1-4)GlcNAc(β)-OMe (107,9 mM)	No data available	0,7	No data available	Nilsson et al. (1993)
Fucosyl-α-1,3-*N*-acetylglucosamine	*Lactobacillus casei* α -L-fucosidase AlfB (50 U)	*p*NP-fuc (50 mM)	GlcNAc (200 mM)	No data available	No data available	23	Díaz et al. (2013)
Fucosyl-α -1,6-*N*-acetylglucosamine	*Lactobacillus casei* α -L-fucosidase AlfC (100 U)	*p*NP-fuc (50 mM)	GlcNAc (200 mM)	No data available	No data available	56	Díaz et al. (2013)
6-fucosyl-N-acetyllactosamine	Porcine liver α -L-fucosidase (0,45 U)	Fucα-*p*NP (27,3 mM)	LacNAc (347,8 mM)	3,9	9	3	Murata et al. (1999)
2-acetamido-	*Aspergillus niger*	4-nitrophenyl α -L-	2-Acetamido-	17,3	3	58	Ajisaka

2deoxy-3-O-α –L-fucopyranosyl-D-glucose	α-L-fucosidase (0.074 U)	Fucopyranoside (29,2 mM)	2-deoxy-D-glucose (188,4 mM)				et al. (1992)
3-O- α-L-fucopyranosyl-D-glucose	*Corynebacferium* sp. α-L-fucosidase (0.4 U)	4-nitrophenyl α -L-Fucopyranoside (29,2 mM)	D-glucose (231,3 mM)	17,5	3	61	Ajisaka et al. (1992)

[a] Concentration of donor substrate (mM) was calculated from mass and final volume referred to the literature of the stated authors as well as from the molecular weight of each donor substrate

[b] Concentration of acceptor substrate (mM) was calculated from mass and final volume referred to the literature of the stated authors as well as from the molecular weight of each acceptor substrate

According to table 2 it seems to be obvious that mainly α-L-fucosidases of various origins are used in the in vitro synthesis of HMO.

In particular syntheses with α-L-fucosidases from *Alcaligenes* sp., *Penicillium multicolour* or *Lactobacillus casei* were responsible for high yields of HMO or core sructures thereof. Especially 3`-fucosyllactose containing structures could be synthesized in high yields whereby α-L-fucosidases from *Alcaligenes* sp. (yield of 67% ; Baer et al. 1980) and *Penicillium multicolour* (yield of 49% ; Ajisaka et al. 1998) should be noted for further engineering strategies.

Further on the in vitro synthesis of glucosamines by α -L-fucosidase AlfC from *Lactobacillus casei* (yield of 56% ; Díaz et al. 2013) and the in vitro synthesis of milk disaccharides 2-acetamido-2deoxy-3-O-α–L-fucopyranosyl-D-glucose (yield of 58% ; Ajisaka et al. 1992) as well as 3-O- α-L-fucopyranosyl-D-glucose (yield of 61% ; Ajisaka et al. 1992) by α-L-fucosidase from *Aspergillus niger* may be convenient for further engineering strategies in order to improve amounts of HMO respectively oligosaccharide based structures.

Although the production of fucosylated HMO is generally achievable by in vitro approaches, whole cell catalysts based on fucosyltransferase activity may be rather convenient in order to enable a less cost intensive and more efficient synthesis of carbohydrate structures at large scale (Ruffing and Chen 2006) as can be seen on the example of the in vivo synthesis of 2`-fucosyllactose in a study by Baumgärtner et al. (2013).

In vivo synthesis of fucosylated human milk oligosaccharides (HMO)

Different studies reveal that synthesis of human milk oligosaccharides is actually feasible by the heterologous overexpression of bacterial fucosyltransferase genes in various metabolically engineered *Escherichia coli* strains so that production of 2`-fucosyllactose and Lewis X oligosaccharides is still possible at double-digit gram range (Baumgärtner et al. 2013, Koizumi et al. 2000).

Furthermore it should be mentioned that it is principally possible to synthesize 2`-fucosyllactose through overexpression of human fucosyltransferase genes in transgenic rabbits and mice. These laboratory animals might be generally adapted for HMO production since they show very simple milk oligosaccharide profiles facilitating the detection and quantization of (neo-) oligosaccharides (Prieto et al. 1997; Prieto 2012) but yields are rather low compared to the heterologous overexpression of bacterial fucosyltransferase genes in *Escherichia coli* so far.

For instance concentration of 2`-fucosyllactose was beneath 1 g/L when produced in transgenic mice (Prieto et al. 1997) whereas a formation of 2`-fucosyllactose in even double-digit gram quantities could be obtained in metabolically engineered *Escherichia coli* (Baumgärtner et al. 2013; Koizumi et al. 2000).

Therefore this review focuses on the in vivo production of fucosylated HMO structures by different *Escherichia coli* strains.

An efficient in vivo synthesis of fucosylated HMO in *Escherichia coli* often relies on an adequate metabolic engineering strategy with focus on an active expression of fucosyltransferase genes on the one hand and the provision of donor GDP-fucose as well as internalization of lactose (Baumgärtner et al. 2013) or N-acetylglucosamine based acceptors (Koizumi et al. 2000) on the other hand.

An *Escherichia coli* based in vivo system with two engineered metabolic pathways called salvage synthesis pathway and de novo synthesis pathway was developed in a study by Baumgärtner et al. (2013) in order to produce 2`-fucosyllactose as illustrated in figure 3:

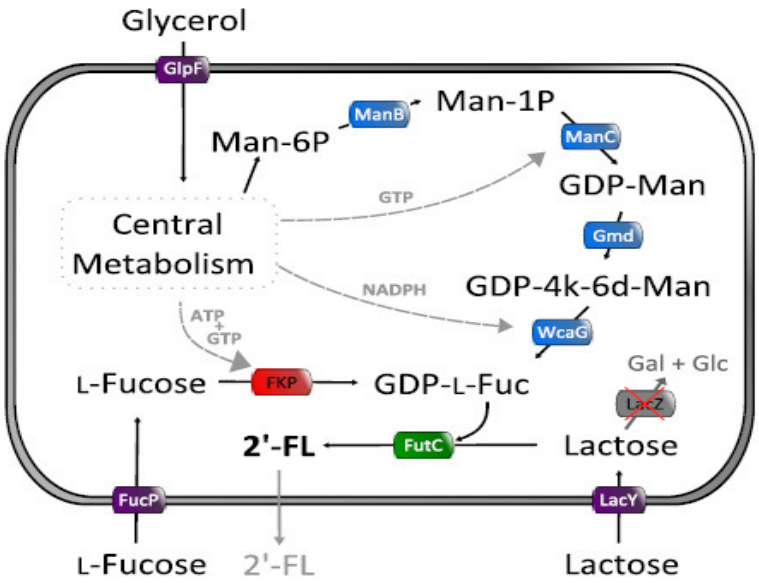


Fig. 3 Metabolic pathways I for the in vivo synthesis of 2`-fucosyllactose (2`-FL) in *Escherichia coli* adapted from Baumgärtner et al. (2013). Red: Salvage synthesis pathway. Blue: De novo synthesis pathway. Green: Fucosyltransferase FutC. Molecules and enzymes are abbreviated as follows: Gal, D-galactose. GDP-4k-6d-Man, GDP-4keto-6-deoxymannose. GDP-Man, GDP-α-D-mannose. GDP-L-Fuc, GDP-β-L-fucose. Glc, D-glucose. FucP, fucose permease. GlpF, glycerol MIP channel. LacY, lactose permease. Man-1P, α-D-mannose-1-phosphate. Man-6-P, α-D-mannose-6-phosphate. ManB, phosphomannomutase. ManC, mannose-1-phosphate guanylyltransferase. Gmd, GDP-mannose 4,6 dehydratase. WcaG, GDP fucose synthase. FutC, α1,2-fucosyltransferase. LacZ, β-galactosidase

For Central Metabolism and GDP-fucose synthesis glycerol was taken up by the cell through the transport protein glycerol MIP channel.

Glycerol could be converted to α-D-mannose-6-phosphate (Man-6-P) which was used to form GDP-L-fucose by enzymes of the de novo synthesis pathway including phosphomannomutase ManB, mannose-1-phosphate guanyltransferase Man C, GDP-mannose 4,6-dehydratase Gmd and GDP-fucose synthase WcaG as can be seen in figure 3.

Glycerol was used as a substrate for Central Metabolism to provide cofactors ATP, GTP and NADPH that are essential for the de novo- or salvage synthesis pathway (Baumgärtner et al. 2013; Lee et al. 2012).

The uptake of lactose by lactose permease LacY enabled the synthesis of 2`-fucosyllactose with 2`-fucosyltransferase FutC whereas internalisation of L-fucose via fucose permease FucP was responsible for a further GDP-fucose formation by the salvage synthesis pathway supporting 2`-fucosyllactose production.

Additionally these metabolically engineered *Escherichia coli* cells were deficient in LacZ activity to avoid the consumption of lactose to galactose and glucose by the enzyme betagalactosidase LacZ and to synthesise 2`-fucosyllactose instead.
Furthermore degradation of intracellular fucose which is also needed in 2`-fucosyllactose formation was prevented by knocking out the genes for fuculose-kinase and fucose-isomerase through insertion of genes *fkp* of the salvage synthesis pathway or a second fucosyltransferase *futC* gene.
Genes coding for fucosyltransferase FutC from *Helicobacter pylori* and for enzymes of the de novo synthesis pathway and salvage synthesis pathway from figure 3 could be implemented via Lambda-Red recombineering technique and insertion of expression cassettes in *Escherichia coli* (Baumgärtner et al. 2013).
This integration of expression cassettes turned out to be a promising alternative to an approach adapted from Lee et al. (2012) which included the transformation of expression plasmids instead and lactose as the sole carbohydrate resource, causing the conversion of more than 90 % of lactose to other products than GDP-L-fucose and 2`-fucosyllactose.
Baumgärtner et al. (2013) could show that a strain including both, the salvage synthesis and de novo synthesis pathway was able to synthesise almost twice as much 2`-fucosyllactose than a strain with a de novo synthesis pathway only which reveals that a further salvage synthesis pathway significantly improved 2`-fucosyllactose yields in *Escherichia coli*.
Also a second copy of the *futC* gene had a positive, but smaller effect on 2`-fucosyllactose yield in *Escherichia coli.*
Therefore a fed batch fermentation with an *Escherichia coli* strain with a salvage synthesis gene *fkp* as well as a second fucosyltransferase gene *futC* showed the highest 2`-fucosyllactose yield of nearly 400 mg g_{CDW}^{-1} closely followed by a strain with a salvage synthesis gene *fkp* but just one copy of *futC* (Baumgärtner et al. 2013).
2′-fucosyllactose synthesis with *Escherichia coli* JM109 gwBC-F2 was performed in a 30 l stirred-tank reactor with a batch volume of 8.4 l and a total feed volume of 5.1 l consisting of a certain salt medium and glycerol as carbon source.
Once glycerol (26.42 g/l) from batch medium was consumed and the expression of fucosyltransferase gene *futC* induced by 0,5 mM IPTG, 629.08 g/l glycerol and 150 g/l lactose were fed to 8 liters of batch medium which had been inoculated with 0.4 liters over-night preculture. Feeding was performed at a temperature of 37°C and a regulated pH of 7,0 so that a final 2`-fucosyllactose concentration of 20,28 ± 0,83 g/L was feasible as can be seen in table 3.

Since NADPH is widely consumed in biomass and production of metabolites like xylitol (Lee et al. 2011) but is also essential for the synthesis of GDP-fucose by enzyme GDP-fucose-synthase WcaG of the de novo synthesis pathway (Baumgärtner et al. 2013), it is useful to increase the energy pool.

Whereas Baumgärtner et al. (2013) increased the energy pool by feeding the cell with glycerol there is a further strategy based on the overexpression of genes coding for NADPH generating enzymes.

According to Sauer et al. (2004), mainly enzymes from tricarboxylic acid (TCA) cycle, oxidative pentose phosphate pathway (PPP) as well as the proton-translocating and membrane-bound transhydrogenase PntAB of the anaplerotic pathway are associated with effective NADPH-generation since 35–45%, 20–25% and 35–45% of the NADPH can be produced by enzymes of PPP, TCA cycle and transhydrogenase PntAB of the anaplerotic pathway within the cell.In a study by Lee et al. (2011) it was shown that GDP-L-fucose synthesis in *Escherichia coli* was increased by overexpression of genes for endogenous NADPH regenerating enzymes isocitrate dehydrogenase (Icd) of the TCA cycle, glucose-6-phosphate dehydrogenase (G6PDH) of the oxidative PPP as well as NADP+-dependent malate dehydrogenase (MaeB) of the anaplerotic pathway whereby overexpression of glucose-6-phosphate dehydrogenase (G6PDH) showed the best effect on GDP-L-fucose production. Lee et al. (2011) could obtain a concentration of more than 230 mg GDP-L-fucose / L in a fed batch process based on a pH-stat glucose feeding of an *Escherichia coli* strain which simultaneously overexpressed genes for NADPH regenerating glucose-6-phosphate dehydrogenase (G6PDH) and genes of the de novo synthesis pathway from figure 3. It was demonstrated that both, NADPH regeneration and a sufficient pH dependent carbon supply might be important for a successful GDP-L-fucose production.

In addition Duan et al. (2010) could proof that an increase in GDP-L-fucose yield by glucose-6-phosphate dehydrogenase (G6PDH) respectively NADPH regeneration is associated with a reduced acetate formation of 75% revealing that a decrease of acetate within the cell might support GDP-L-fucose yield since acetate is known to have negative effects on product yields (Duan et al. 2010).

Alternatively or additionally to the mentioned enzyme candidates that can be used for NADPH regeneration in GDP-L-fucose- or 2`-fucosyllactose production, direct conversion of NADH to NADPH through direct phosphorylation could stand for a further and even more attractive alternative to lift the intracellular NADPH concentration and productivity in

microbial in vivo processes because high amounts of NADH from glycolysis are available within *Escherichia coli* to be converted to NADPH (Lee et al. 2013).
According to Lee et al. (2013) NADP+ and NADPH levels could be increased by 13 % respectively 33 % by overexpression of the so called NADH kinase *Pos5p* gene from *S. cerevisiae* in *Escherichia coli* whereas NAD+ and NADH levels could be lowered by 21 % respectively 20 % resulting in an increase of GDP-L-fucose concentration of 51 %.
To sum up, yields of GDP-L-fucose respectively 2`-fucosyllactose in in vivo expression systems can be improved by the overexpression of genes encoding NADPH regenerating enzymes responsible for a continuous energy supply as was demonstrated in the study by Lee et al. (2011) or by increasing the energy pool of NADPH, ATP and GTP through the internalisation of a substrate in the form of glycerine which can be converted in Central Metabolism and is not used for product formation (Baumgärtner et al. (2013).
Furthermore the expression of a positive regulator protein called RcsA in the colanic acid synthesis pathway as well as the inactivation of genes encoding enzymes that are involved in the GDP-fucose conversion to other products than HMO might be appropriate to increase GDP-L-fucose formation within metabolically engineered *Escherichia coli* strains (Lee et al. 2012).
Table 3 gives an overview about possible biotechnological in vivo syntheses of important fucosylated HMO:

Table 3 In vivo production of fucosylated HMO

Bacterial-strains / mammals	*Fucosyltransferases*	*Source of enzyme*	*Substrate*	*Cultivation*	*Desired product(s)*	*Concentration product(s) (g / L)*	*Reference*
E. coli JM 109	α 1,2-fucosyltransferase FutC	*Helicobacter pylori*	Lactose	Fed batch	2`-FL	20,28 ± 0,83	Baumgärtner et al. (2013)
E. coli JM 109DE3	α 1,2-fucosyltransferase FutC	*Helicobacter pylori*	Lactose	batch	2`-FL	1,23 ± 0,011	Lee et al. (2012)
Transgenic mice	FucTi	Human	lactose	-	2`-FL	0,711	Prieto et al. (1997)

Transgenic rabbit	FucTI	Human	lactose	-	2`-FL	No data available	Prieto (2012)
Col1FΔlacZDE3	FutC	*Helicobacter pylori*	lactose LNnT	Fed batch Fed batch	2´-FL LNnFPI	No data available	Drouillard et al. (2006)
E. coli NM522 *C.ammoniagenes*	α 1,3-fucosyltransferase	*Helicobacter pylori*	LacNAc	batch	Lewis X	21	Koizumi et al. (2000)
Col1FΔlacZ	α 1,3-fucosyltransferase FutAon	*Helicobacter pylori*	LNnT Lactose	batch	LNnDFHII 3`-FL	1,7 0,5	Dumon et al. (2004)
Col1FΔlacZ	α 1,3-fucosyltransferase FutB	*Helicobacter pylori*	LNnT	batch	LNnFPII LNnFPV	0,26 0,28	Dumon et al. (2004)

According to table 3 particularly 2`-fucosyllactose and Lewis X oligosaccharides could be efficiently synthesized by -fucosyltransferases FutC and α 1,3-fucosyltransferase from *Helicobacter pylori* in order to obtain concentrations of more than 20 g / L HMO (Baumgärtner et al. 2013; Koizumi et al. 2000).

In contrast to 2`-fucosyllactose other fucosylated HMO such as lacto-N-neo-fucopentaose I (LNnFP I) and V (LNnFP V) have only been produced at concentrations of 250 mg /L (Dumon et al. 2004) yet which reveals that further metabolic engineering might still lie ahead.

In Figure 4 a concentration profile of synthesized HMO from table 3 is illustrated which illustrates the large differences in HMO concentrations obtained from microbial in vivo synthesis so far:

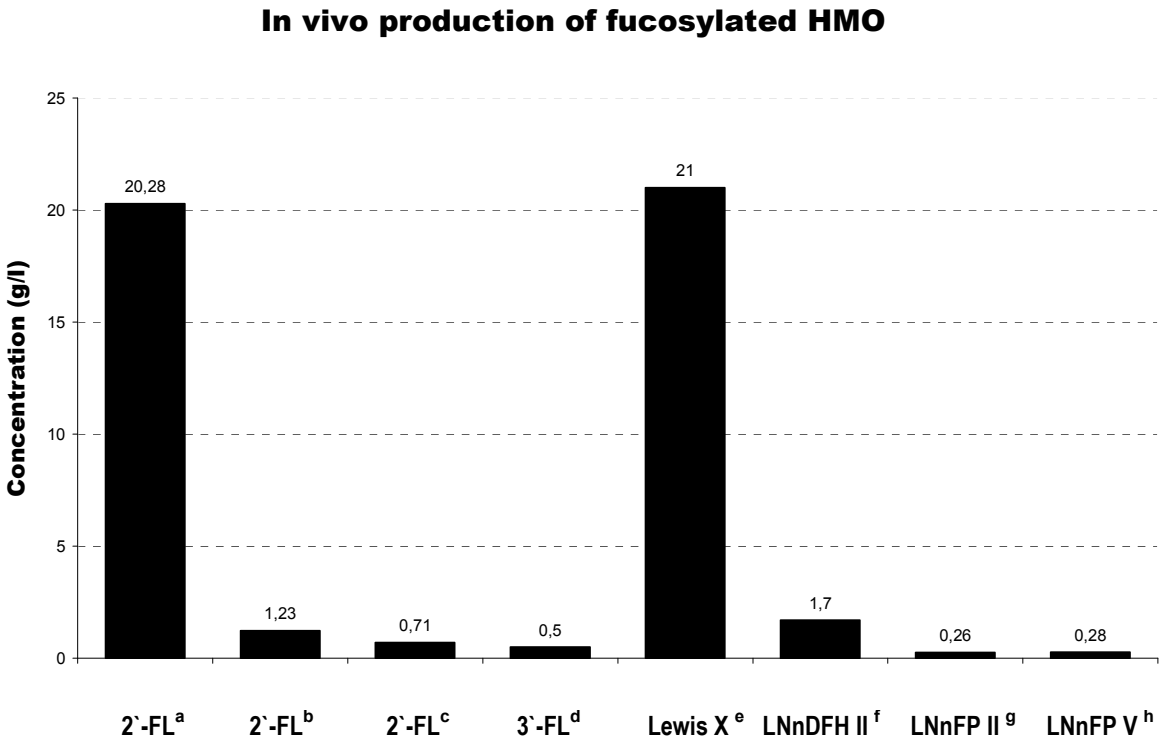


Fig. 4 In vivo synthesis of fucosylated HMO. Concentrations adapted from [a]Baumgärtner et al. (2013), [b]Lee et al. (2012). [c]Prieto et al. (1997), [d]Dumon et al. (2004), [e]Koizumi et al. (200), [f]Drouillard et al. (2006), [g]Dumon et al. (2004), [h]Dumon et al. (2004)

Perspectives of human milk oligosaccharide synthesis and infant formula research

The biotechnological synthesis of fucosylated human milk oligosaccharide (HMO) plays an important role in society.
On the one hand certain Lewis antigen harbouring mothers rely on the production of those HMO because they are rare or not synthesized in their body during lactation (Thurl et al. 2010), on the other hand there are mothers that do not want to breastfeed their babies.
Thus mainly the production of 2`-fucosyllactose may be important for future since 2`-fucosyllactose is peculiarly known to protect infants against certain infections caused by microbial pathogens.
Especially metabolic engineering of *Escherichia coli* will probably be in demand for future because recent results have indicated that various *Escherichia coli* strains are adapted to enable an economical synthesis of carbohydrate structures such as 2`-fucosyllactose or Lewis X oligosaccharides in double digit gram quantities (Baumgärtner et al. 2013;Koizumi et al. 2000) whereas in vitro approaches generally show lower HMO product yields so far as was demonstrated on the in vitro synthesis of HMO by fucosyltransferase- fucosidase- or fucosynthase technology.
Furthermore it cannot be excluded that also transgenic animals might be adapted for a prospective in vivo HMO production at large scale.
It is already known that they show very simple milk oligosaccharide profiles facilitating the detection and quantization of (neo-)oligosaccharides (Prieto et al. 1997; Prieto 2012).
However, with the exception of Lewis X oligosaccharides other N-acetylglucosamine based fucosylated HMO have been produced by *Escherichia coli* in vivo production systems at significantly lower concentrations (Dumon et al. 2004; Drouillard et al. 2006) yet which demonstrates that an optimisation in metabolic engineering or reaction conditions still lies ahead in order to synthesise appropriate amounts of these HMO.

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Chapter II

Literature research Biotechnological production of fucosylated human milk oligosaccharides (HMO) and core structures thereof

Introduction

Human milk modulates the gut microbiota of breastfed infants and represents the predominant diet of newborn infants. It is unique in its complex composition and high concentration of oligosaccharides [1].

Human milk oligosaccharides (HMO), especially fucosylated ones, are essential to protect infants against certain microbial pathogens since in the first year of life a stable population of microflora is established in the human gut as a complex ecology that includes the intestinal mucosa and more than 400 interdependent species of bacteria. HMO have no direct nutritive value to the infant but are substrates for the development of the intestinal microflora and the mucosal immune system [2].

Therefore they may provide a prebiotic effect which enhances colonization by *Bifidobacterium bifidum* [3;4] and to a lesser extent by lactobacilli [5] to care for an intestinal flora.

Alternatively they inhibit the colonization and growth of pathogens competitively in order to protect the cell surface of the new borne [1].

Advances in glycomics have enabled precise determination of milk glycan structures and identification of the specific glycans, especially fucosylated HMO consumed by various gut microbes [1]. In addition genomic analysis of bifidobacteria from infants has revealed specific genetic loci related to milk oligosaccharide import and processing which might be an

evidence for coevolution between the human host, milk glycans, and the microbes they enrich [1].

During lactation there exist big qualitatively and quantitatively differences in fucosylated HMO profiles of female milk according to Lewis blood group inheritance and secretor status which indicates the missing of essential fucosyloligosaccharides in some women [6;7]. Since these HMO are important for infant development they should be added through infant formula if the mother is not able to produce them sufficiently.

The missing of sufficient quantities and purities for in vitro and clinical studies complicates the exact characterization of certain HMO [1].

However, some companies try to mimic mother's milk and add oligosaccharides to the formula in order to obtain the same effects on gastro-intestinal colonisation and pathogen prevention as natural mother's milk does [8].

Recently metabolic engineering has been recognized as a successful tool in the development of whole-cell biocatalysts for oligosaccharide and polysaccharide synthesis.

Advantages of the synthesis process based upon these microbial in vivo systems compared to in vitro approaches are that high average product concentrations can be achieved and no expensive starting materials are needed [9].

No enzymes and cofactors such as NADPH or GTP must be added for synthesis reactions because they can be regenerated within the cell. Therefore this whole cell approach may be more realistic for industrial, large scale applications than the enzymatic in vitro process because it does not require enzyme isolation, expensive cofactors or necessary but limited elements for oligosaccharide synthesis [8].

Microbial synthesis of oligosaccharides and polysaccharides means an energy-intensive and a carbon-intensive process which interacts with the central metabolism and implicates challenges in metabolic engineering and host selection. In contrast to nicotinamide cofactors, the required sugar nucleotides such as GDP-fucose in the production of 2`-fucosyllactose are products of multiple interacting pathways that often must be metabolically engineered [10].

There are different in vivo production systems used in practice with metabolically engineered *Escherichia coli* cells as preferred host strains for the production of 2`-fucosyllactose, the most frequent fucosylated human milk oligosaccharide beside lacto-N-fucopentaose I observed in mother milk [11;12].

These systems are based on two main metabolic pathways: The de novo synthesis pathway and the salvage synthesis pathway that are both applicable in the production of GDP-L-Fucose, an intermediate in the synthesis of 2`-fucosyllactose [13].

Fucosyltransferase FucT2 from *Helicobacter pylori* and alternative fucosyltransferases from other bacterial origins [14] are able to synthesize 2`-fucosyllactose.
They belong to the stereo- and regio-selective α1,2-fucosyltransferases that are able to attach a fucosyl-group in α-linkage to the 2-position of a galactosyl-residue of a saccharide and are used in 2`-fucosyllactose production as a reason [13;14].
Also more complex fucosylated human milk oligosaccharides can be efficiently synthesized through in vivo production systems that are based on *Escherichia coli* strains [15;16] as well as on transgenic animals [12].
In humans, milk oligosaccharides are produced exclusively in the mammary gland and only during lactation, which makes it difficult to study their biosynthetic pathway [17].
However, understanding how HMO are constructed in the human mammary gland gives some indication of enzymes that are needed in the biotechnological in vitro or in vivo production to synthesize certain HMO. Thus the identification and characterisation concerning functionality and substrate specificity of different human fucosyltransferases in the mammary gland is useful to produce human milk oligosaccharides in in vivo production systems that are based on human fucosyltransferase activity in transgenic animals.
One disadvantage of animal in vivo systems is that oligosaccharides in human milk are distinct from those of other species with respect to quantity and diversity, which limits the use of animal models to mimic HMO metabolism but also absorption, pharmacokinetics, and function [17].
Two classes of fucosylating enzymes are frequently used in the biotechnological synthesis of 2`-fucosyllactose and fucosylated-complex human milk oligosaccharides (HMO), the so called fucosyltransferases and transfucosidases.
Former are important in the synthesis of HMO in humans [18] respectively in transgenic mammals [11] and are further responsible for fucosylating processes in prokaryotes [19].
They lay the foundation in the in vivo production of HMO [13] and can be used for in vitro experiments too [14;20].
Latter are applied solely in vitro to form human milk oligosaccharides (HMO) so far since it was demonstrated that they show only hydrolizing but no fucosylating activity in vivo [21].
It should be mentioned that alternative methods to in vivo or in vitro systems can be used to obtain HMO amounts:
One alternative is the recovery or isolation of HMO from natural resources and other species than humans, especially mammals such as primates, cows, pigs, goats, sheep or elephants by membrane and filtration technologies:

It has been already shown that HMO-like oligosaccharides were separated by membrane technology on a large scale from by-products in goat cheese production and that a two-stage tangential ultrafiltration–nanofiltration system could be used to separate oligosaccharides from goat milk [22].

There exist also the opportunity to go back to chemical syntheses to produce HMO: Automated solid-phase oligosaccharide synthesis [23] can be applied to produce HMO fast and efficiently as can bee seen in the productions of complex oligosaccharides such as the Lewis X or Lewis Y oligosaccharides that can be performed within one day. The oligosaccharides being synthesized through this method are linked to insoluble material like beads or resins that allow the fast separation of reaction products from excess reagents, soluble reaction by-products, and solvents [24].

Further on a relative new approach of a crystalline intermediate technology was found to assist a purification step in protection and deprotection of saccharides which enabled the fast synthesis of 2′-O-fucosyllactose [25].

Although the development of chemical synthesis leads to improvements such as higher purification, velocity or amounts of HMO, several hurdles for the industry-scale synthesis of HMO as well as negative aspects such as low stereoselectivity, low overall yields, and the use of toxic reagents that are not suitable for food products (infant formula) are still known [26].

To sum up there exist different methods that can be applied in the production respectively isolation of fucosylated HMO:

First the in vivo bacterial or animal production systems, second enzymatic in vitro approaches, third the recovery or isolation of HMO from natural resources and fourth chemical syntheses whereas the first two methods seem to be the most promising in production of fucosylated HMO at large scale and are therefore accurately described in this diploma thesis.

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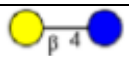
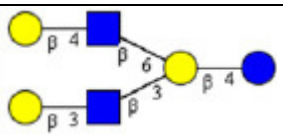
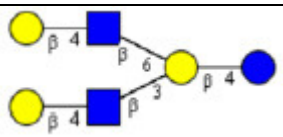
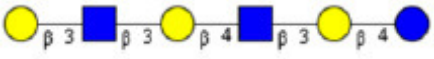
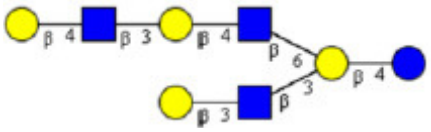
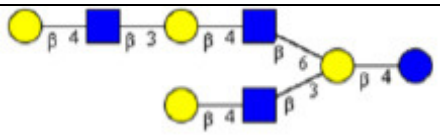
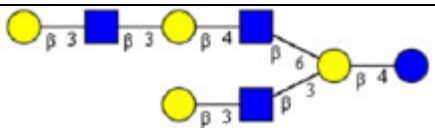
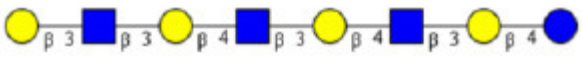
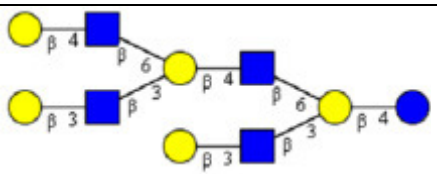
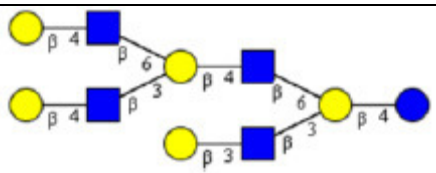
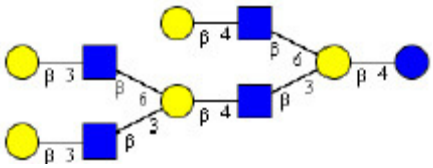
Lewis antigen dependent structures, functional features and occurence of fucosylated human milk oligosaccharides (HMO)

Human milk consists of the 4 substantials lactose, which is the most frequent component, lipids, especially triglycerides, proteins such as caseins and complex lactose based oligosaccharides that are varying between 10 and 20 g/L milk [27;28].
It is unique in its complex composition and high concentration of oligosaccharides that are quantitatively the third largest and most diverse component of breast milk after lactose and lipids.
So far, more than 130 unique structures of human milk oligosaccharides (HMO) were characterized [18;29] and experimental evidence for the existence of a minimum of 100 additional species has been reported [30;31;32].
They have no direct nutritive value to the infant but are substrates for the development of the intestinal microflora and the mucosal immune system.
Therefore they may provide a prebiotic effect which enhances colonization by *Bifidobacterium bifidum* [3;4] and to a lesser extent by lactobacilli [5] to care for an intestinal flora or inhibit the colonization and growth of pathogens respectively.
Bifidobacterium bifidum digests lactose and produces acetic acid and lactic acid whereas Lactobacilli are also capable of hydrolysing lactose but produce lactic acid as only end product. The growth of many other microorganisms is suppressed by the acidic conditions created through *Bifidobacterium bifidum* [3;4] and Lactobacilli [5] in the intestine of babies which might protect them from intestinal infections.
Because human milk oligosaccharides are structural similar to structures of the epithelial cell surface glycoconjugates and show homology to erythrocyte glycoconjugates that determine Lewis blood group types [18;33], they have been also proposed to inhibit the binding of pathogenic viruses and bacteria competitively.
HMO serve as soluble ligand analogs to pathogenic viruses and bacteria through their glycoconjugate structure respectively epitops and thus can bind them. They block pathogen adhesion to the cell surface and help to protect breast-fed infants against infections.
Human milk oligosaccharides (HMO) inhibit binding of several pathogens such as *Escherichia coli, Vibrio cholera, Shigella, Salmonella* or *Campylobacter jejuni* [17].

As a reason there is a high demand in the biotechnological production of human milk oligosaccharides (HMO), especially fucosylated oligosaccharides to avoid diseases from children and replace breast feeding through infant formula milk.
Different effects caused by various HMO are summarized in table 6 [33].
A high variation of infant formula milk content is required since it is known that HMO composition can vary among individuals and over the course of lactation:
Differences in HMO structures concerning size or sequence are associated with the individual diverging Lewis blood types and the secretor status.
Additionally amounts of HMO are influenced by the activity of essential fucosyltransferases or fucosidases during lactation [4;34].
In breast feeding milk largest differences during lactation can be observed in concentrations of products synthesised by α1,2-fucosyltransferases which leads to the assumption that the expression level of major α1,2-fucosyltransferase FucTII is mainly responsible for alternative oligosaccharide patterns observed in humans [4;6;18;34].
Human milk oligosaccharides (HMO) are thought to be composed of two main groups: First neutral oligosaccharides that consist of galactose, N-acetylglucosamine, fucose, and a lactose core, second anionic or acid oligosaccharides containing the same oligosaccharide compositions with N- acetylneuraminic acid at α2-3- or α2-6- positions of galactose, GlcNAc or glucose [34]. HMO typically contain a lactose moiety at the reducing end and a fucose at the non reducing end.
Nearly all examined human milk oligosaccharides (HMO) that appeal as antigens or prebiotic stimuli for bacteria such as *Bifidobacterium bifidum* or lactobacilli are built up by single or multiple elongations of lactose with two different types of *N*-acetylglucosamine units:
The dominant type 1 chains (Gal(β1-3)GlcNAc) or type 2 units Gal(β1-4)GlcNAc respectively LacNAc [11] which are called N-lactosamines.
Both form either linear or branched structures as can be seen in table 1.
Table 1 shows different lactose based core structures [33], table 2 neutral HMO with their antigenic determinants [11;18;27].

Table 1 Various core structures found in human milk oligosaccharides adapted from Blank et al. [33]. Yellow circles, galactose. Blue circles, glucose. Blue squares, N-acetylglucosamine

Cores	Structure[a]
Lactose	
LNT (Lacto-N-tetraose)	
LNnT (Lacto-N-neotetraose)	
LNH (Lacto-N-Hexaose)	
LNnH (Lacto-N-Neohexaose)	
Para-Lacto-N-hexaose	
Para-Lacto-N-neohexaose	
Lacto-N-octaose	
Lacto-N-neooctaose	
Iso-Lacto-N-octaose	
Para-Lacto-N-octaose	
Lacto-N-decaose	

Lacto-N-neodecaose	
Inverse-Lacto-N-neodecaose	

[a] core structures found in human milk oligosaccharides adapted from Blank et al. [33]

Structures of important neutral milk oligosaccharides were adapted by Kobata et al.[18] and Kunz et al. [27] and are depicted in table 2 [11].

Table 2 Important structures of oligosaccharides found in human milk

Name	Abbreviation[a]	Structure[b]
Lactose	Lac	Galβ1-4-Glc
2`-fucosyllactose	2`-FL	Fucα1-2Galβ1 -4-Glc
3`-fucosyllactose	3`-FL	Galβ1-4(Fucα1-3)Glc
Difucosyllactose	DFL	Fucα1-2Galβ1 -4(Fucα1-3)Glc
Lacto-N-fucopentaose I	LNFP I	Fucα1-2Galβ1 -3GlcNAcβ1-3Galβ1-4Glc
Lacto-N-fucopentaose II	LNFP II	Galβ1 -3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc
Lacto-N-fucopentaose III	LNFP III	Galβ1 -4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc
Lacto-N-difucohexaose I	LNDFH I	Fucα1-2Galβ1 -3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc
Lacto-N-difucohexaose II	LNDFH II	Galβ1 -3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc

[a] Abbreviations were cited in Castanys Muñoz et al. [11] ; [b] Structures are adapted from Castanys-Muñoz et al. [11], Kobata et al. [18] and Kunz et al. [27]

Neutral HMO can show α1,2-glycosidic bound fucoses at terminal galactose residues, α1,3-glycosidic bound fucoses at a glucose or N-acetylglucosamine residue as well as α1,4-glycosidic bound fucoses at a N-acetylglucosamine residue only.
Human milk contains 4 mayor α1,2-fucosylated neutral oligosaccharides [4;18] called lacto-N-fucopentaose I (LNF-I or synonymous LNFP I), lacto-N-difucohexaose I or synonymous difucosyl lacto-N-tetraose I (LND- I or synonymous dFLNTI), lactodifucotetraose or synonymous difucosyllactose (LD or synonymous dFL) and finally 2`-fucosyllactose (2`FL)

which represents the main component of fucosylated human milk oligosaccharides beside lacto-N-fucopentaose I [4;18;35].

Beside α1,2-fucosylated oligosaccharides, human milk contains three mayor neutral oligosaccharides with α1,3-and α1,4- linkages: 3`-fucosyllactose (3`FL), lacto-N-fucopentaose II (LNF-II or synonymous LNFP II) and III (LNF-III or synonymous LNFP III) as well as lacto-N-difucohexaose II or synonymous difucosyl lacto-N-tetraose II (LND-II or synonymous dFLNT II) [4;18;35].

There are also human milk oligosaccharides (HMO) in human milk that can not be classified in neutral fucosylated oligosaccharides but belong to acid HMO:

3`-sialyllactose, 6`-sialyllactose, Lst-a, Lst-b and Lst-c [36;37].

Representative concentrations of some important oligosaccharides in human milk are depicted in table 3 [12]:

Table 3 Averaged disaccharide (lactose) and oligosaccharide concentrations found in human milk

Name	Structure[a]	Concentration (mg/L)[b]
Lactose	Galβ1-4-Glc	50000
2`-fucosyllactose	Fucα1-2Galβ1 -4-Glc	200
Lacto-N-fucopentaose I	Fucα1-2Galβ1 -3GlcNAcβ1-3Galβ1-4Glc	200
Lacto-N-fucopentaose II	Galβ1 -3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc	20
Lacto-N-fucopentaose III	Galβ1 -4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc	50
Lacto-N-tetraose	Galβ1 -3GlcNAcβ1-3Galβ1-4Glc	400
Lacto-N-difucopentaose I	Fucα1-2Galβ1 -3(Fucα1-4) GlcNAcβ1-3Galβ1-4Glc	25
Lacto-N-neotetraose	Galβ1 -4GlcNAcβ1-3Galβ1-4Glc	60

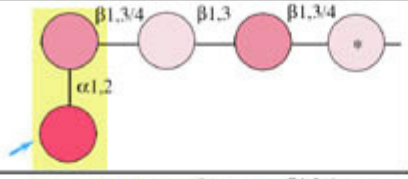
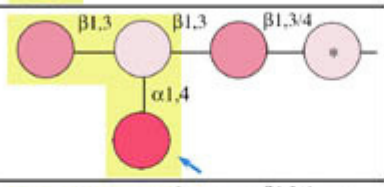
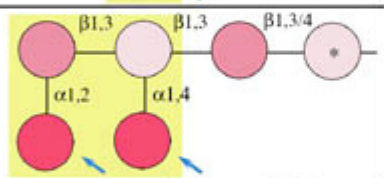
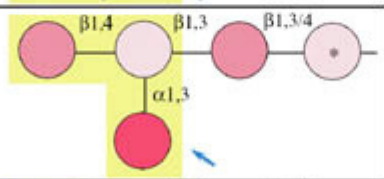
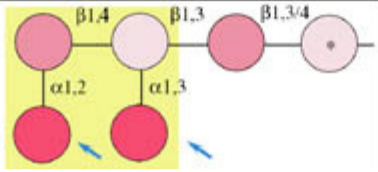
[a] Structures adapted from Prieto et al [12] ; [b] Concentrations cited in Prieto et al. [12]

According to table 3 2`-fucosyllactose and lacto-N-fucopentaose I represent the most frequent fucosylated human milk oligosaccharides with a concentration of about 200 mg / L observed in mother milk [12].

Because neutral human milk oligosaccharides (HMO) are structural similar to structures of the epithelial cell surface glycoconjugates and show homology to erythrocyte glycoconjugates

that determine H and Lewis blood group determinants, they have been proposed to inhibit the binding of pathogenic viruses and bacteria competitively [17].
Lewis and H determinants are depicted in table 4 [38]:

Table 4 Structure of H- and Lewis determinants adapted from Blood Group Antigen Gene Mutation Database [38]. Pink circles, galactose. Red circles, fucose. Lightish rose, GlcNAc. Yellow shade, core (minimal determinant) structure. Blue arrows indicate the possibility of different carbohydrate chains that can be connected to fucose

Phenotype	Structure	Minimal determinant structure
H		Fuc–α1→2–Gal–β1–R
Le^a		Gal–β1→3 GlcNAc–β1–R Fuc–α1→4
Le^b		Fuc–α1→2–Gal–β1→3 GlcNAc–β1–R Fuc–α1→4
Le^x		Gal–β1→4 GlcNAc–β1–R Fuc–α1→3
Le^y		Fuc–α1→2–Gal–β1→4 GlcNAc–β1–R Fuc–α1→3

The Lewis antigen or blood group system is based on two genes from chromosome 19: The so called *FUT3* or Lewis gene (*Le* gene) and the *FUT2* or secretor gene (*Se* gene). Both genes are expressed in the mammary gland and can contain either dominant alleles *Le* and *Se* coding for fucosyltransferases FUT3 and FUT2, respectively, or recessive alleles *le* and *se* that are not functional. Two main types of human milk oligosaccharides are known that describe the Lewis antigen system: Lewis antigens Lewis a (Le-a or synonymous Le^a) or Lewis b (Le-b or synonymous Le^b) that are based on the described type 1 chains. They are the basis of 3

different individual phenotypes that can occur in the antigen or blood group system of a human: Le(a+b-), Le(a-b+) or Le(a-b-) [39].
It is possible that the prevalence in white individuals averages 22% for Le(a+b-) , 72% for Le(a-b+) and 6% Le(a-b-) [40;41] although there exist different data that deviate marginally as can be seen in a study by Thurl et al. [6].
It should be mentioned that there is a phenotype and variant of "non secretor" Le(a-b-) namely Le(a-b-c+d-) examined by Thurl et al. [6] but its occurrence was very rare compared to the other types with a prevalence of 1% in white individuals as shown in table 5.
All Lewis antigens are red cell antigens that are not produced by the erythrocyte itself. Instead, Lewis antigens are components of exocrine epithelial secretions that come from the mammary gland for example, and are subsequently adsorbed onto the surface of the erythrocyte [38].
To understand the applicability of Lewis dependent human fucosyltransferases in the biotechnological transgenic synthesis of certain fucosylated HMO in animals [35;42] it is helpful to know about their corresponding and correlating features determined in humans. Thus the following pages give some indication of their functionality and substrate specificity observed in humans. They are also auxiliary to understand features of bacterial fucosyltransferases that are homologous to human ones and used in heterologous expression in bacteria.
Additionally they give an overview about functionality and substrate specificity of various human fucosyltransferases in in vitro experiments compared to in vivo observations. Furthermore it will be explained in which human Lewis antigen phenotypes these fucosyltransferases emerge since it is essential to know where the fucosyltransferase harbouring genes can be isolated from in order to use them for transgenic production systems. On the other site the Lewis antigen dependent absence of different fucosylated HMO forms a basic understanding why certain Lewis antigen harbouring mothers rely on the production of HMO that are rare or not synthesized in their body during lactation.
Three different types of Lewis- dependent fucosyltransferases are mainly responsible for the fucosylation of core structures in human milk oligosaccharides and are important for the production of several human milk oligosaccharides in transgenic animals [35;42] as well as for in vitro experiments as accurately described in chapters 2 and 3.
Fucosyltransferase III (FucT III or synonymous Fut3), fucosyltransferase II (FucTII or synonymous Fut2) and various α1,3 fucosyltransferases (FucT IV,VI,VII and IX or synonymous Fut4,5,6,7 and 9) [19;33].

Fucosyltransferase III (FucT III) is a fucosyltransferase with α1-4 fucosyltransferase activity encoded by the Lewis (*Le*) gene. In vivo it catalyzes the transfer of GDP-activated fucose in α1-4-linkage to subterminal *N*-acetylglucosamine on type 1 chain Gal(βl,3)GlcNAc in glycoconjugates. Thereby it converts the precursor oligosaccharide type 1 which is called Lacto-N-tetraose (LNT) to the Le-a antigen.

This enzyme shows also an α1-3 fucosyltransferase activity on type 2 chain Gal(βl,4)GlcNAc of precursor oligosaccharide Lacto-N-neotetraose (LNnT) which leads to the formation of the Le-X and Le-Y antigen [19;33] whereby its specificity for type 2 chains is smaller [19].

In vitro the Lewis (Le) gene-encoded Fucosyltransferase III (FucT III) might also use both the type-1 and type-2 chain substrates Gal(β1-3)GlcNAc respectively Gal(β1-4)GlcNAc and thus may be involved in the production of the Le-a and Le-b antigens through its α1-4 activity as well as in synthesis of antigens Le-X and Le-Y through its α1-3 activity [43].

According to Oriol et al. [44] Fucosyltransferase III (FucT III) prefers type-1 substrate Gal(β1-3)GlcNAc in vitro too.

In a female who represents the *Le* allele respectively shows FucT III activity and is a "non-secretor" (Lewis a+b-) because she is homozygous for the *se* allele, the Le-a antigen will be present in her bodily fluids (milk) and on her erythrocytes.

These individuals synthesize and secrete Le-a antigen but not antigen Le-b. They are able to produce A, B and H antigens (H antigen type 2) but do not secrete them:

With very rare exceptions H determinants type 2 are present on all human red cells such as erythrocytes, or in plasma. But H determinants type 1 are absent or expressed very weakly in secretions unless a human also carries a dominant secretor gene *Se* [45].

A person who carries both, the dominant *Le* as well as *Se* allele (Lewis a-b+) has exocrine cells in the mammary gland that show also Fucosyltransferase II (FucTII) beside Fucosyltransferase III (FucTIII) activity. Through the combined action of these two enzymes the H determinant type 1 is first synthesized from type 1 precursor Lacto-N-tetraose (LNT) with Fucosyltransferase II (FucTII) and then converted to Le-b with Fucosyltransferase III (FucTIII) in people that carry the dominant *Le* as well as *Se* allele.

There is no Le-a antigen found on erythrocytes or in plasma in people that show both Fucosyltransferase II (FucTII) as well as Fucosyltransferase III (FucTIII) although it is found beside Le-b antigens in secretions such as human milk [33].

Fucosyltransferase II (FucT II) encoded by the secretor gene Se is an α1-2 fucosyltransferase which in vivo prefers the transfer of fucose in α1-2-linkage to terminal galactose on type 1

chain Gal(βl,3)GlcNAc rather than to terminal galactose on type 2 chain Gal(β1-4)GlcNAc in glycoconjugates.
The product resulting from α1-2-linkage to terminal galactose on type 1 chain is the so called H antigen type 1.
Also a third type of glycoconjugate which does not contain the GlcNAc residue is efficiently fucosylated by this enzyme which is the type 3 chain or acceptor Galβ1,3GalNAc [19].
According to Oriol et al. [44] the enzyme FucT II is also more active on type 1 chain than on type 2 chain in vitro.
People with Lewis b antigen and α1-2 fucosyltransferase FucT II activity show the phenotype Le(a-b+) and represent secretors since A, B and H antigens can be produced and secreted beside Le-b.
Lewis negative people (Le(a-b-)) are homozygous for the recessive *le* allele and can be either secretors that show the phenotype Le(a-b-) or non-secretors with a phenotype Le(a-b-c+d-) which shows a significant lower rate than the other three Lewis blood types as can be seen in table 5 [46]. In the case of an inactive FUT3 both epitopes will be obliterated and that is why the phenotype is called Le(a-b-) and is lacking determinants Le^a and Le^b, irrespective of the activity state of FucT II. Thus, the introduction of the alpha 1,2 fucose by a functional Fuc TII in the absence of an alpha 1,4 adjacent fucose does not result in the Lewis epitopes but yields H antigens type 1.
This phenotype also describes a secretor because antigens H, A and B can be secreted. Especially donors with active secretor gene do secrete the H antigen [46].
The expression of Lewis epitopes on the erythrocytes is dependent on adsorption from plasma of Lewis-bearing glycolipids. As a reason the Lewis negative phenotype may not occur uniquely as a result of an inactive allele, but may result from several other causes such as a failure of adsorption to the erythrocyte or presence of an allele whose product is less active or may be selectively inactive towards glycolipid precursor substrates [38].
Together 80% of the Europeans -including secretion positive Le(a-b-)- as well as Le(a-b+)-donors- secrete ABH substances in saliva and other secretions and are typed as Se [33;40;41].
There is also a second form of α1,2-fucosyltransferase called Fucosyltransferase FucT I encoded by the H gene which preferences both the type 1 and type 2 acceptors respectively chains and is less efficient in fucose transfer to Galβ1,3GalNAc (type 3) [19;45;47;48].
The α1,2-Fucosyltransferase FucT I encoded by the H gene is able to transfer fucose in α1-2-linkage to terminal galactose on the type 2 chain Gal(β1-4)GlcNAc in glycoconjugates. The product of this synthesis is the so called H antigen type 2 [45].

Liu et al. [45] found that H enzyme activity was similar for both type 1 and type 2 precursors Galβ1-3GlcNAcβ1- and Galβ1-4GlcNAc β1-, respectively, whereby the *Se* encoded enzyme FucT II activity was about 6 times higher on type 1 precursor compared to type 2 precursor. Expression of the H type 1 antigen in mucous cells was recognized to be dependent on the *Se* gene, but expressions of the H type 1 and H type 2 antigens in striated and interlobular duct cells such as mammary gland cells showed dependence on the H gene although the possibility of additional *Se* gene expression in duct cells could not be completely excluded.
With very rare exceptions H determinants type 2 are present on all human red cells such as erythrocytes, but H determinants type 1 are absent or expressed very weakly in secretions unless a human also carries a dominant secretor gene *Se*. The H determinant type 1 is most observed in secretions of Lewis negative donors with phenotypes Le(a-b-) and an active *Se* gene because it can not be converted to Lewis Le-b through the missing activity of FucT III. It is also found in Le(a-b+) [45;49].
The Lewis blood group dependent occurrence of main fucosyltransferases FucT III (α1-2 FucT) and FucT II (α1-4 FucT) and antigens with their core structure is resumed in table 5 [46].

Table 5 Characteristics of Lewis blood groups Lewis blood groups vary in enzyme expression, carbohydrate epitope and relative percentage in population

Lewis blood group	Expressed enzyme	Involved gene	Percentage of population[a]	Carbohydrate epitope
Lewis (a-b+)	α1-2 FucT α1-4 FucT	Se/- ; Le/-	69	Fucα1-2Galβ1-3GlcNAcα1-4Fuc
Lewis (a+b-)	α1-4 FucT	se/se ; Le/-	20	Galβ1-3GlcNAcα1-4Fuc
Lewis (a-b-)	α1-2 FucT	Se/- ; le/le	5-10	Fucα1-2Galβ1-3GlcNAc
Lewis (a-b-c+d-)	-	Se/se *; le/le*	1	Galβ1-3GlcNAc

[a] Percentage of population adapted from Kunz et al. [27]

As a third type of fucosyltransferases there are various α1-3 fucosylating α1-3 fucosyltransferases which can be observed in all Lewis blood group donors in contrast to the α1-4 Fucosyltransferase FucT III which is only expressed in Lewis blood group positive humans with either a blood group Lewis (a-b+) or Lewis(a+b-) and at least one active gene locus for FucTIII [19].

α1-3 fucosyltransferases attach fucose in α1-3-position either to the glucose residue or *N*-acetylglucosamine of the oligosaccharide precursor. Thus the formation of Le^x and Le^y epitopes is mainly performed by them although FucTIII is also able to produce Le^x and Le^y.

α1-3 fucosyltransferases FucTIV–VII and FucTIX as well as α1-3 / α1-4 FucTIII might all be able to synthesize Le^x and Le^y epitopes in vivo [19;50].

The role of the α1-3 -FucTs IV and VII in synthesizing Le^x and Le^y epitopes seems to be proven because their corresponding genes are highly conserved among mammals and contribute to the formation of selectin ligands [51].

Beside the 3 types Fucosyltransferases FucT III, FucT II and various α1,3 fucosyltransferases that might be mainly involved in Lewis and H antigen based HMO synthesis there are also other fucosyltransferases found in human and tested on their substrate specificity in vivo or in vitro:

Although FucT III has been considered as the main enzyme in humans producing α1,4-fucosylation [52;53], there is some evidence suggesting that FucT V also can fucosylate type 1 precursors in vitro [54;55] and in vivo [19;56;57].

In addition to fucosyltransferase FucT II fucosyltransferase FucT I was further identified to direct α1,2-fucosylation on type I structures [45].

It has been demonstrated in vitro that the specificity of fucosyltransferases can differ from specificity observed in vivo which can be seen on FucT I and FucT V that prefer type 2 to type 1 in vitro [44;52].

FucT VIII was characterized as an α1,6-fucosyltransferase which transfers fucose to asparagine linked GlcNAc structures in hepatoma cells [58] whereas gene *pofut1* encodes an O-fucosyltransferase (POFUT1) which adds fucose directly to polypeptide chains [59]. POFUT1 is expressed in human tissues such as spleen, thymus, prostate, testis, ovary, small intestine or colon and peripheral blood leukocytes.

Two additional α1,3-fucosyltransferase genes, *fucT X* and *fucT XI*, have been recognized in the human genome by comparison with fucosyltransferase sequences in the Drosophila melanogaster genome [60].

FucT X was identified as α1,3-fucosyltransferase in the ventricular zone of the embryonic brain to maintain stem cells in an undifferentiated state [61] and was also found in other tissues such as skin, small intestine, liver, kidney, lung or muscles of fetus and adults [62].

Whereas classical monoexonic α1,3/4-fucosyltransferases such us Fut 3-7 and 9 that are also present in human milk prefer short lactosaminyl acceptors, the polyexonic FUT10 enzymes

use mainly biantennary *N*-glycans linked to glycopeptides or to biotin aglycone as substrates. Linear short acceptors cannot be used by FUT10 and FUT11 to transfer α-fucose. The substrate acceptor pattern of FUT11 may be similar to FUT10 fucosyltransferases according to Mollicone et al. [62].

Enzymes FucT VIII, POFUT1, FucT X and FucT XI might not occur in human milk and thus it can be assumed hat they do not play a role in the production of HMO.

FucT IV, VI , VII and IX were characterized as pure α1,3- fucosyltransferases that fucosylate type 2 structures whereas FucT III and FucT V show both, an α1,3 fucosylating activity on type 2 chains and an α1,4 fucosylating activity on type 1 chains [19;33;50;56;57].

Figures 1 and 2 give an overview about possible in vivo syntheses of neutral HMO based type 1 or type 2 chains by different fucosyltransferases [33]. They does not include synthesis of type 1 based structures by FucT V which might also show α1,4 fucosylating activity as well as synthesis of α1,2-fucosylated type 1 and type 2 based structures by α1,2-fucosyltransferase FucT I.

Figure 1 shows the synthesis of the HMO core structure lactose in the Golgi of the mammary gland [33]:

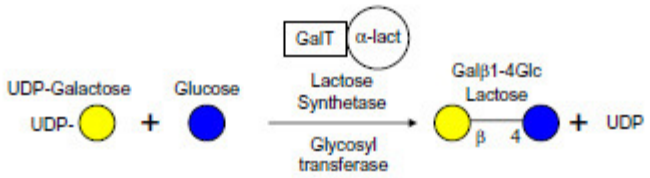


Figure 1 Biosynthesis of lactose in mammals adapted from Blank et al [33]. Yellow circle, UDP-galactose. Blue circle, glucose. Abbreviations: GalT, galactosyltransferase. α-lact, α-lactalbumin. Galβ1-4-Glc, lactose. UDP, Uridine-diphospho galactose

The monosaccharide galactose (Gal) is transferred from UDP-galactose to glucose via glycosyltransferases.

In mammals this is performed by galactosyltransferases and α-lactalbumin.

Figure 2 shows the 3 processes that are important in the production of Lewis and H determinants as well as branched oligosaccharide structures from the monosaccharide lactose: Elongation and fucosylation that are essential for the production of Lewis and H determinants and elongation, branching and fucosylation that are important for branched structures.

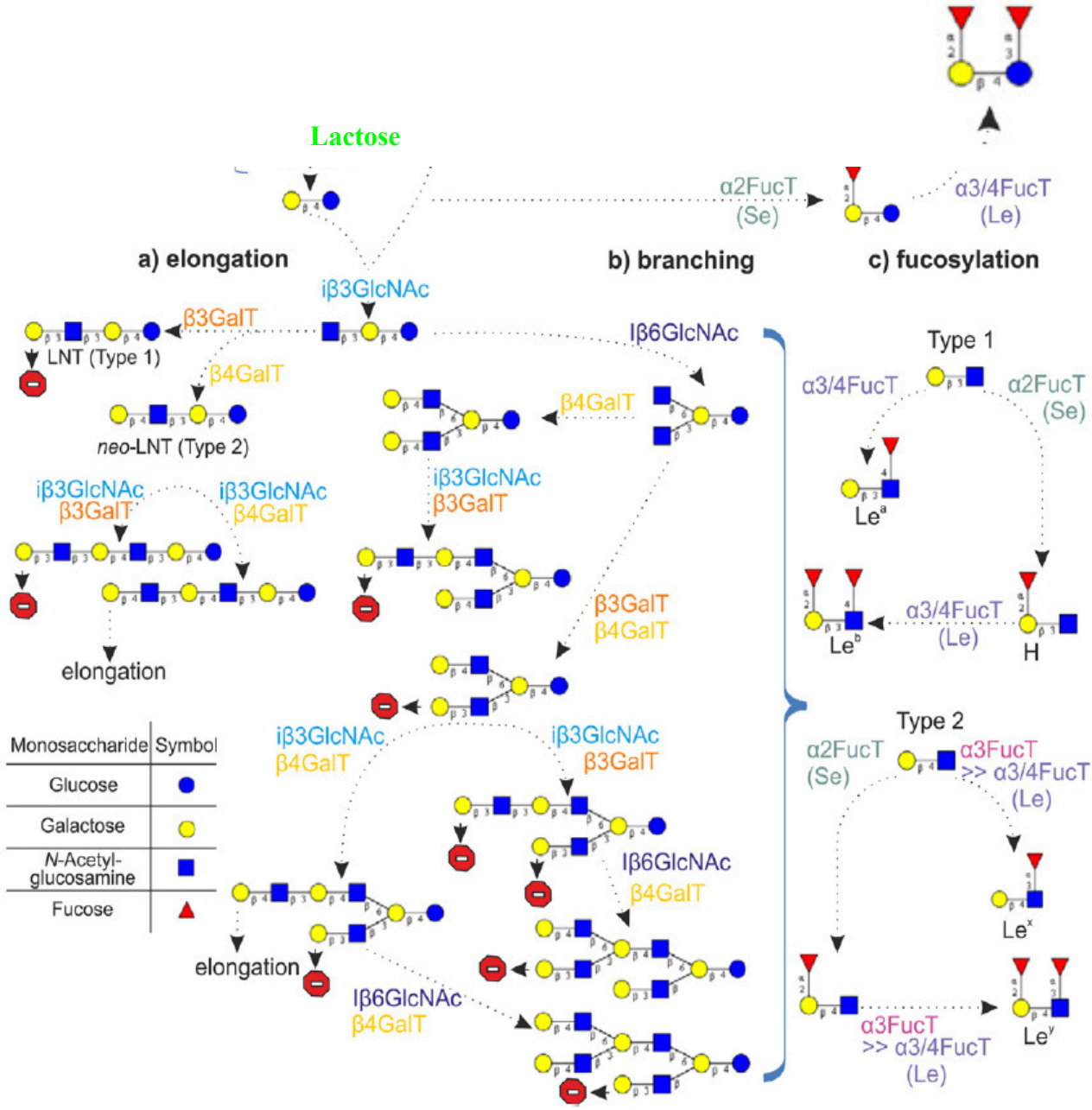


Figure 2 Biosynthesis of neutral complex human milk oligosaccharides (HMO) adapted from Blank et al [33]

N-acetylglucosaminyltransferase iβ3GlcNAcT adds N-acetylglucosamine (GlcNAc) in the β1–3 position to terminal galactose (Gal) whereas N-acetylglucosaminyltransferase iβ6GlcNAcT attaches GlcNAc in β 1–6 position to terminal Gal and is involved in branching. Gal can be attached by galactosyltransferase β3GalT to GlcNAc in β 1–3 position or by galactosyltransferase β4GalT in β 1–4 position, respectively.

The fucosyltransferase α2FucT (FucT II or synonymous Fut2) encoded by the *Se* gene attaches fucose (Fuc) in α 1–2 position to terminal galactose (Gal).

Various α3FucTs (α1,3-fucosyltransferases) transfer fucose in α1–3 position to GlcNAc and α3/4FucT (FucT III or synonymous Fut3) attaches fucose in the α1–3/4 position to GlcNAc and in the α 1–3 position to the glucose (Glc) of the lactose molecule. The no entry signs describe that no additional elongation occurs [33].

Figure 3 includes the fucosylation pathways only but describes the synthesis of alternative glucose analogues of Lewis determinants additionally.

The Lewis synthetic pathway in figure 3 adapted from Newburg et al. [2] describes alternative pathways of 2`-, 3`- and 4`- fucosyloligosaccharides:

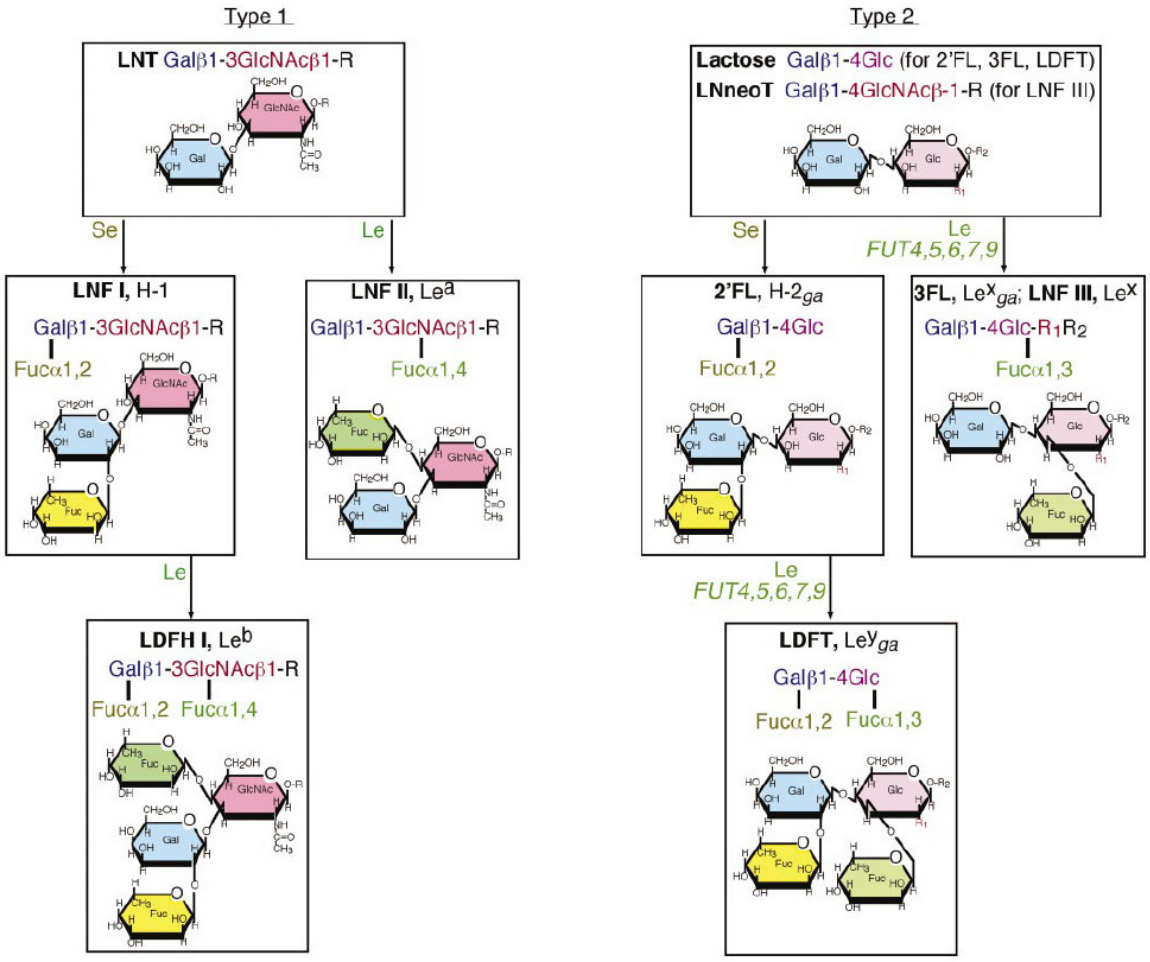


Figure 3 Proposed synthetic pathways of important fucosyloligosaccharides of human milk adapted from Newburg et al. [2]

The principal type 1 fucosyloligosaccharides are synthesized from LNT through fucosylation on the Galβ1,3GlcNAc residue on the terminal end of lactose (-R). LNT can serve as substrate for fucosyltransferase III (Le), a product of the *FUT3* (*Le*) gene, to produce LNF-II, which is a pentasaccharide carrying the Le-a group . On the other side LNT can be converted to LNF-I, a pentasaccharide carrying the H-1 group (type 1 H antigen), by fucosyltransferase II (Se), a product of the *FUT2* (secretor) gene. LNF-I can be used by fucosyltransferase III (Le) to synthesize LDFH-I or synonymous LND-I, a hexasaccharide carrying the Le-b group.
The principal type 2 fucosyloligosaccharides are synthesized mainly from precursor lactose, but also from precursor lacto-N-neotetraose, that is, Galβ1,4GlcNAc on a lactose terminus. Synthesis from lactose results in glucose analogs (ga) of these Lewis antigens, whereby ±R2 is ±H and ±R1 is ±OH [2].
Lacto-N-neotetraose can be elongated by fucosyltransferases III (Le), IV, V, VI, VII, or IX through their α1,3 fucosylating activity to LNF-III, a pentasaccharide carrying the Le-x group. Lactose on the other side can be converted to 3`-fucosyllactose (3`-FL), the glucose analog of the Le-x pentasaccharide , Le-x ga. Alternatively, lactose can serve as substrate for fucosyltransferase II (Se) to synthesize 2`-fucosyllactose (2`-FL), the glucose analog of H-2 (Type 2) pentasaccharide respectively H antigen type 2 [2].
2`-FL can be acted on by fucosyltransferase III (Le) or perhaps fucosyltransferases IV, V, VI, VII, or IX to form LDFT or synonymous LD (Lactodifucotetraose), the glucose analog of Le-y hexasaccharide [61].
As a result of the Lewis blood group dependent occurrence of fucosyltransferases different concentrations of fucosylation patterns were found in Lewis Le(a-b-), Le(a+b-) and Le(a-b+) donors by Thurl et al. [6] as can be seen in figure 4:

Concentration

[mmol / L]

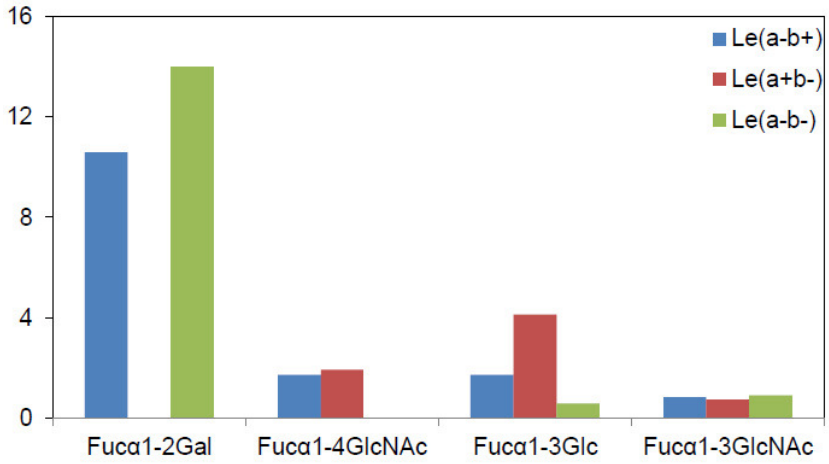


Figure 4 Different concentrations of fucosylation patterns in dependence of Lewis blood group found in mature human milk adapted from Thurl et al. [6]. Fucα1-2Gal, α1-2-fucosylated terminale galaktose residues; Fucα1-4GlcNAc, α1-4-fucosylated subterminale N-acetylglucosamines; Fucα1-3Glc, α1-3-fucosylated reducing glucose; Fucα1-3GlcNAc, α1-3-fucosylated subterminale N-acetylglucosamine

According to figure 4 it is obvious that large differences in fucosylated HMO concentrations between Le(a-b-) and Le(a-b+) donors are based on the Lewis-gene dependent expression of α1,4-fucose molecules that are only found in females with Le(a-b+) or Le(a+b-).
Furthermore a Le(a+b-) donor shows a significant lower viability in neutral HMO mainly because of the missing of those oligosaccharides that all contain an α1,2-fucose in their structure and that can not be synthesized since the enzyme FucTII is not active.
As a result 2′-FL can not be detected in milk samples obtained from individuals of nonsecretor blood type Lewis Le(a+b-) [7].
Observational studies indicate that certain HMO from a secretor are associated with various preventive antiadhessive effects on *Campylobacteror, Calciviruses, E.coli, Shigella*, *Salmonella, Vibrio Cholerae or HIV-I* as well as with promoting effects on intestinal maturation in preterm neonates by *bifidobacteria* [2;33;63].
Diarrhea caused by *Calcivirus* can be significantly reduced through LDFH-I binding whereas diarrhea by *Campylobacteror jejuni* is lowered by higher 2`-FL concentrations [64].
The incidence of *Campylobacter* diarrhea in breast-fed infants depends on the amount of 2`-fucosyllactose respectively oligosaccharides which contain H type 2 epitopes in mother milk

[17] It could be demonstrated that α 1,2-fucosylated HMO decreased *Campylobacter jejuni* infections in mice [65] and significantly prevented diarrhea in breast-fed infants [17].
In earlier studies an inhibition of the adherence to the intestinal epithelial cells by even L-fucose was described for 14 different *Campylobacter* strains from human and birds [66].
In addition to calciviruses a resistance against Stable Toxin producing *Escherichia coli* was reported through the presence of α1,2- linked fucosylated oligosaccharides [67] and according to Morrow et al. [64] inhibition of *calciviruses* could be associated with Le^b epitopes.
Lewis specific HMO such as α1,2-fucosylated HMO might perform protective effects against urinary tract infections in babies [33;68] and adults [40;41]. Uropathogenic *E. coli* (EPEC) have been identified to bind glycolipids in "non secretor" women, causing more urinary tract infections than in secretors, in whom the receptor is masked by the additional α1–2-Fuc [69].
Newburg et al. [2] were able to demonstrate that also infections by enterotoxigenic *E.coli* (ETEC) could be prevented through feeding babies with fucosyloligosaccharide rich milk whereas babies provided with milk with a lower fucosyloligosaccharide content showed the symptoms of enterotoxigenic *E.coli*.
It was shown in mice and in in vitro experiments that an α1,2-fucosylated oligosaccharide could bind the diarrhea causing toxin called stable toxin (ST) of ETEC [70].
Harris B et al. [71] reported that persons with blood group O were less likely than those with other blood groups to become infected with *V. cholerae* O1 whereas a protection against *V. cholerae* O139 could not be proven [67].
Additional Crane et al. [67] observed an increased severity of cholera among patients with blood group O once infected with both the O1 and O139 serogroups of *V. cholerae, probably* due to the fact that there is no interference of the H antigen based A and B histo-blood group glycoconjugates in blood group O individuals with the binding of Cholera Toxin CT to its intestinal receptor, the ganglioside GM1 [72].
While both Cholera Toxin (CT) and heatlabile enterotoxin (LT) of enterotoxigenic *Escherichia coli* (ETEC) bind to the GM1 receptor, it was proven in vitro that, in contrast to CT, LT can additionally adhere to the blood group A antigen as an alternate receptor through which the activation of cyclic AMP can be induced [73]. This in vitro observation indicates that individuals with blood group O show a higher risk of severe cholera but not of enterotoxigenic *Escherichia coli*-associated (ETEC) diarrhea infections [74].
Some HMO show haptenic activities of blood group determinants: The α1,2 fucosylated structures 2′-FL and LNF-I show haptenic activity of the H determinants type 2 and 1, respectively.

LND-I shows activity of the Le^b determinant, and LNF-II activity of the Le^a determinant [18]. Concerning their resistance it can be said that donors with the Lewis(a-b+) blood group might be more resistant against pathogens such as *Helicobacter pylori* and *Norwalk virus* compared to Lewis(a+b-)and (a-b-)- donors because Le(b) as well as H antigen based structures 2′-FL and LNF-I are receptors for them and inhibit their colonization and growth at the cell surface [18].

Moreover it was reported that O blood group donors show a higher susceptibility to *Norwalk virus* [75].

To sum up Lewis negative people with the "non secretor" Le(a-b-) phenotype and thus an absence of 2`-fucosylated HMO are exposed to an increased risk for infections by uropathogenic *Escherichia coli* [2;67] *Campylobacter jejuni* [63;65] or *Vibrio cholerae* [65]. Moreover, "non secretor" status respectively the lack or lower concentration of α1,2-fucosylated structures might play a key role in mortality, necrotizing enterocolitis [63] or Crohn's disease [76] in preterm infants as well as in infections by *Haemophilus influenza* and *Candida albicans* [64].

Table 6 summarizes some important effects of Lewis and secretor gene-related factors [46]:

Table 6 Effects of oligosaccharides with Lewis core structures adapted from Blank et al [46]

Effect	Factor	Investigated in
In vitro		
Bifigogenic	Lewis a, type 1 H trisaccharide	*Bifidobacterium longum ssp. Infantis*
Antiadhesive	HMO, ia. Lewis epitope bearing	*Campylobacter jejuni, E. coli, Vibrio cholerae, Shigella, Salmonella, HIV-1*
Antiadhesive vs. *C.jejuni*	α1-2-Fuc-HMO	Carcinoma-derived human epithelial cells
Ex vivo		
Antiadhesive vs. *C.jejuni*	α1-2-Fuc-Lac, neutral HMO	Fresh human intestinal mucosa
In vivo		
Colonization with *C.jejuni* reduced	- α1-2-Fuc-oligosaccharides	Pups of transgenic nice

Preventive vs. diarrhea from *C.jejuni*, calcivirus	α1-2-Fuc-HMO	Infants
Preventive vs. diarrhea from *E.coli*	α1-2-Fuc-HMO	Infants
Association with mortality, gram-negative sepsis and necrotizing enterocolitis	Low or non secretor status	Preterm infants
Association with Crohn`s disease	Nonsecretor status	Pediatric/adult individuals

Recently it was shown in mice by Zhu et al. [77] that human milk oligosaccharide Lacto-N-fucopentaose III (LNFP III) which contains the Le-x trisaccharide is able to cause a decrease in the severity of autoimmune encephalomyelitis (EAE) and to reduce Central Nervous System (CNS) inflammation through modulation of the innate (humoral) and adaptive T cell based immune system:

LNFP III might induce NO synthesis in cooperation with pro- inflammatory interferone IFN-γ and granulocyte-macrophage colony-stimulating factor GM-CSF by nitric oxide synthase 2 in splenic inflammatory monocytes (IMCs) in humans. Through NO synthesis T cell proliferation may be suppressed as could be shown in vitro and in mice. A higher expression of several immune regulatory enzymes such as arginase-1 in splenic inflammatory monocytes (IMCs) was also caused by LNFP III treatment in vitro. Higher Arginase-1 concentrations may correspond to an activation of macrophages by LNFP III [78].

Additionally the treatment of IMCs with LNFP III had a positive effect in reducing the production of pro-inflammatory cytokines or chemokines compared to treatment with lipopolysaccharides (LPS).

Furthermore it was demonstrated in an ex vivo experiment that it upregulates the synthesis of anti-inflammatory (regulatory) cytokines interleukin IL 10 of IMCs and TH2 and the synthesis of TH2 specific cytokines in a splenocyte. Thereby it lowers the ratio of IFN-γ to anti-inflammatory (regulatory) cytokines although it was observed that IFN-γ expression was also moderately raised.

Last but not least it might lower migration by dendritic cells across brain endothelium in vivo which reduces EAE severity and CNS inflammation as could be demonstrated in vitro [77].

Because of the Lewis blood group based expression of different fucosyltransferases three oligosaccharide patterns or profiles were found to occur in human milk by Kobata et al. [18] in approximately 50 human milk samples as can be seen in figure 5:

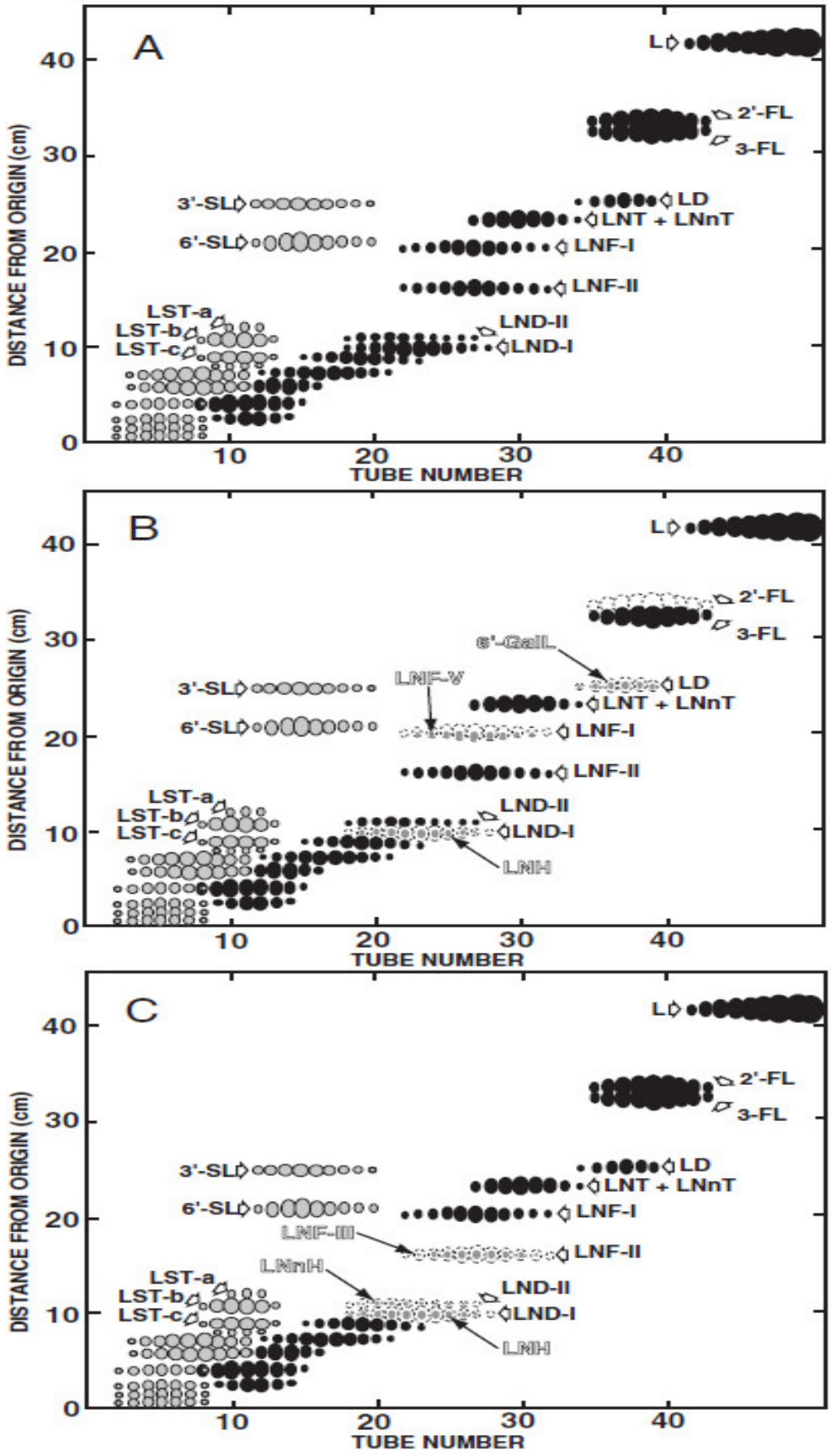


Figure 5 Human milk oligosaccharide (HMO) patterns observed in A (Le $^{a-b+}$), B (Le^{a+b-}) and C (Le^{a-b-}) donors adapted from Kobata et al [18]

About 80% of human milk contained all fourteen oligosaccharides as shown in Fig. 5A. Approximately 15% of human milk lacked four oligosaccharides as shown in Fig. 5B, and remaining 5% lacked three oligosaccharides as shown in Fig. 5C. The missing oligosaccharides were indicated in Fig. 5B and C by dotted lines. The small grey spots, detected at the positions of missing oligosaccharides, represent minor oligosaccharides that are hidden under the major oligosaccharides [18].

The oligosaccharide patterns found in propositi agreed with individual Lewis blood groups: Profile Fig.B represented non-secretors, and those who gave the profile Fig. 5C were all Lewis negative, lacking both Le^a and Le^b antigens. The four oligosaccharides 2′-FL, LD (dFL), LNF-I (LNFP I), and LND-I (dFLNTI), that were missing in the milk of "nonsecretor" individuals with Lewis blood group Le(a+b-), contain the Fucα1-2Gal group in common. This could be an evidence for the absence of fucosyltransferase II in the secretory organs such as the mammary gland of "non secretor"individuals since it is known that fucosyltransferase II (FucTII) is responsible for the formation of the disaccharide group. On the other side the three oligosaccharides LNF-II (LNFP II),, LND-I (dFLNTI) and LND-II (dFLNTII) that all contain the Fucα1-4GlcNAc group could not be observed in Lewis negative individuals Le(a-b-), indicating that this group of females may lack fucosyltransferase III for the formation of the Fuc,1-4GlcNAc group [18].

The mentioned differences in oligosaccharide patterns between Lewis blood groups corresponds to a concentration profile of fucosylated human oligosaccharides (HMO) found in mature human milk in a study by Thurl et al. [6] as can be seen in figure 6.

Furthermore it describes the Lewis blood group specific occurrence of additional oligosaccharides namely fuco-lacto-N-hexaose I (FLNHI), fuco-lacto-N-hexaose II (FLNHII) and difuco-lacto-N-hexaose (dFLNH) that were not analyzed by Kobata et al. [18].

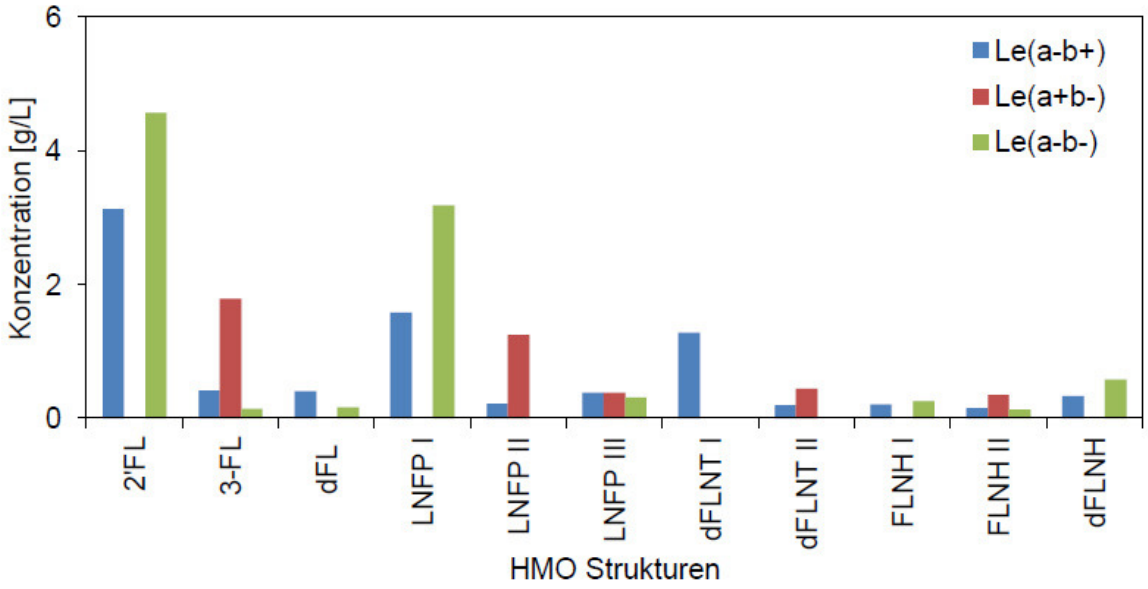


Figure 6 Lewis antigen dependent concentrations of 11 various human milk oligosaccharides (HMO) adapted from Thurl et al. [6]

According to figure 6 it is apparent that a Le(a+b-) donor shows a significant lower viability in neutral HMO mainly because of the missing of oligosaccharides 2'FL, dFL, LNFP I, dFLNT I, FLNH I and dFLNH that all contain an α1,2-fucose as core structure and can not be synthesized since the enzyme FucTII is not expressed.
Large differences in HMO concentrations, especially in LNFP II, dFLNH I und II concentration between Le(a-b-) and Le(a-b+) donors are based on the Lewis-gene dependent expression of α1,4-fucose molecules that are only found in females with Le(a-b+) or Le(a+b-). Variations concerning HMO concentrations between Lewis donors correspond to divergent ratio measurements by Newburg et al. [2] between 2-linked fucosylated oligosacchaides and those fucosyloligosaccharides with 3- and 4-linkages as can be seen in figure 7 adapted from Newburg et al. [2]:

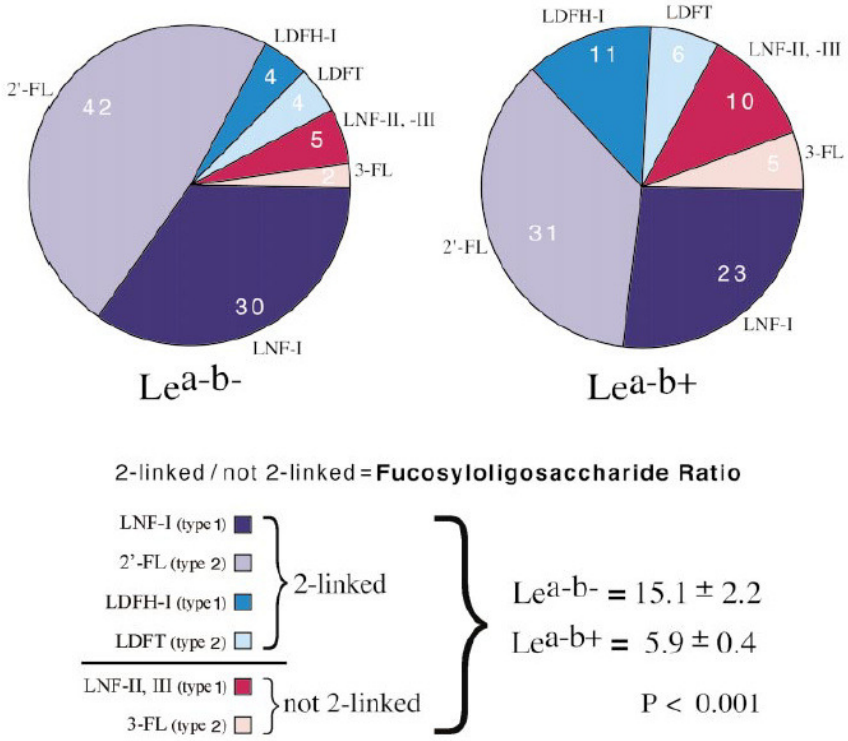


Figure 7 Distribution of fucosylated human milk oligosaccharides (HMO) of different Lewis blood group donors adapted from Newburg et al. [2]

Via Serologic blood typing 91 different Lewis blood group types were analyzed

In figure 7 the relative percentage of oligosaccharides with regard to total milk oligosaccharides is shown for each Lewis blood group type.

Standard errors of the percentage of each oligosaccharide for the milk of Le^{a-b-} milks were equal to or less than 2 whereas those of Le $^{a-b+}$ donors were all equal to or less than 1.

Analysis of variance and Student's t-test were used to check the variations in content of each of the oligosaccharides in milk of both Lewis blood group types.

P value < 0.03 for LDFT, <0.003 for LNF-I, and <0.001 for all others.

The ratios of 2-linked fucosyloligosaccharides to other fucosyloligosaccharides in a milk sample corresponds to the large differences in oligosaccharide concentrations between Le^{a-b+} and Le^{a+b-} donors. A significantly higher ratio (LNF-I + 2`-FL + LDFH-I + LDFT) divided by (LNF-II,-III + 3-FL) was calculated for mothers of the Le^{a-b-} Lewis blood group type compared with Le^{a-b+} mothers [2].

There was a significantly higher value observed in ratio in milk from Le(a-b-) mothers (15,1 ± 2,2) compared to milk produced by Le(a-b+) donors (5,9 ± 0,4) [2] and corresponds to the concentration profile of figure 6 by Thurl et al. [6].

The higher value of the ratio in Le(a-b-) can be explained by a higher amount of the 2`-fucosylated oligosaccharides LNF-1 (H-1) and 2`-FL (H-2) despite a decrease in LDFH-1 (dFLNTI) respectively Le-b hexasaccharide concentration and LDFT (dFL) respectively Le-y hexasaccharide concentration compared with Le(a-b+) donors.
Furthermore a lesser amount of 3`- and 4`- linked fucosyloligosaccharides is responsible for this higher value.
A frequency distribution of the fucosyloligosaccharide ratios from 24 Le(a-b-) and 67 Le(a-b+) mothers by Newburg et al. [2] demonstrates that Le(a-b-) donors might tendentially show higher values in fucosyloligosaccharide ratios as illustrated in figure 8:

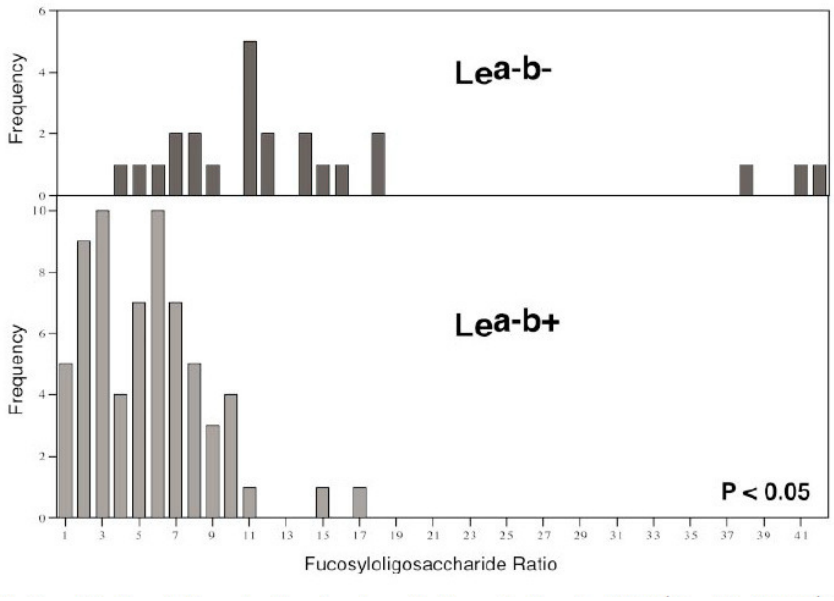


Figure 8 Frequency distribution of Le^{a-b+} and Le^{a+b-} donors adapted from Newburg et al. [2]. Significant differences in distributions were calculated by chi-square analysis ($p<0,05$)

Fucosylated oligosaccharide ratios of Le(a-b+) donors varied between values of 1 and 18 whereas Le(a-b-) mothers showed a range between 4 and 42 [2].
It was also observed that not only large differences in single human oligosaccharide concentration but also total concentration of neutral human milk oligosaccharides depend on the Lewis blood group status.
In the study by Thurl et al. [6] a Le(a-b-) donor shows an average concentration of 10,5 g / L human milk, a Le(a-b+) donor an average concentration of 9.5 g / L human milk and a Le(a+b-) a significantly lower average concentration of only 5,6 g / L human milk.

The absence of the 2`-fucosyltransferase FucTII in Le(a+b-) donors might reveal not only the lowest viability of the three Lewis blood group types but also the significantly lowest value in average concentration of HMO in human milk which substantiates the importance of α1,2-fucosylated oligosaccharide structures.

Since 2`-fucosylated and other HMO prevent babies from diseases [17] or show prebiotic effects [3,4] but are sometimes missing during lactation, biotechnological processes that can produce them efficiently are in demand.

Alternative processes have been developed and are described in the following chapters.

In vivo synthesis of 2`-fucosyllactose and fucosylated-complex human milk oligosaccharides (HMO) by different fucosyltransferases

In vivo synthesis through metabolic engineering of microbial cells and animals represents one of the most promising strategies to obtain high amounts of oligosaccharides such as fucosylated human milk oligosaccharides (HMO) to support (infant formula-) research and clinical development. It is important to achieve high efficiency of synthesis in a single strain to make the process economically viable because this strategy avoids the complication of coupling the oligosaccharide synthesis with central metabolism for energy production by using multiple types of cells, each with a specialized role. Although so far this strategy is known to give higher product concentrations [10].

Since it avoids expensive starting materials and enzyme isolation, once the strain is developed respectively metabolically engineered, it can be efficiently scaled-up in a fermentor to produce the needed quantity of compounds [10]. Product concentrations at gram scale were achieved in HMO producing in vivo systems as can be seen in 2`-fucosyllactose production by the use of metabolically engineered whole cell catalysts that contain certain metabolic pathways [13].

An economic analysis puts the cost of oligosaccharide synthesis through in vivo syntheses at a price range from $30–50$/g product [10,79] which can be regarded as an achievement of metabolic engineering and the basis that many essential oligosaccharides such as 2`-fucosyllactose have now been available in gram scale and at realistic costs.

In contrast to 2`-fucosyllactose other fucosylated HMO such as lacto-N-fucopentaose (LNFP-1), lacto-N-difucohexaose I (LNDFH-I) or lacto-N-neofucopentaose (LNnFP) could have been only synthesized in milligram scale [80] which demonstrates that significant challenges still lie ahead in metabolic engineering and enzymatic in vivo synthesis concerning these structures.

An efficient oligosaccharide synthesis system requires active glycosyltransferases such as fucosyltransferases, a continuous energy supply, the provision of donors and internalization of acceptors [10].

Expression of active glycosyltransferases and engineering success depends strongly on the choice of host strain and the origin from which the enzyme was first isolated.
Fucosylated human milk oligosaccharides (HMO) could be successfully synthesized in bacterial hosts [13] through the heterologous overexpression of bacterial fucosyltransferase genes which showed homology to human ones. Furthermore fucosylated human milk oligosaccharides (HMO) could be synthesized through the overexpression of human fucosyltransferase genes in transgenic mammals [12].
Other constellations like overexpressions of human fucosyltransferase genes in prokaryotes or bacterial fucosyltransferase genes in humans might not be advisable in order to gain high production rates since the genetic differences between pro- and eucaryotes seem to be too high [42].
Usually heterologous genes such as human genes that are coding for certain fucosyltransferases or other enzymes are transformed into the organism instead of homologous genes for different reasons:
On the one hand heterologous genes might not negatively interfere with internal protein synthesis for mammary gland development as homologous genes often do.
On the other hand most experiments are designed in order to produce human primary and secondary transgenic products since there is a high demand on human gene products such as fucosyltransferases or fucosylated HMO [42].
To yield high HMO concentrations within a strain such as *Escherichia coli* it is important to use metabolic engineering in order to obtain higher amounts of GDP-L-fucose, an intermediate of HMO synthesis as well as to overexpress the essential fucosyltransferase(s) which is (are) needed to produce a certain fucosylated human milk oligosaccharide.
In *Escherichia coli* strains stronger GDP-L-fucose and 2`-fucosyllactose production can be either achieved through the overexpression of NADPH regenerating enzymes and enzymes for the so called de novo synthesis pathway or through enzymes that are associated with the so called salvage synthesis pathway [13,81].
Another strategy for enhanced GDP-fucose production is to overexpress a positive regulator protein called RcsA in the colanic acid synthesis pathway and by inactivating enzymes involved in GDP-fucose conversion to other products than 2`-fucosyllactose in order to re-direct the flux of GDP-fucose to oligosaccharide production [81-83].
Beside a sufficient amount of GDP-L-fucose an adequate concentration of the acceptor substrate is important in order to yield high product formation.

An efficient method for the conversion of lactose into lacto-N-neotetraose was achieved by a β –galactosidase negative *Escherichia coli* strain which overexpressed *Neisseria meningitis IgtA* and *IgtB* genes for β1,3-N-acetylgucosaminyltransferase and β1,4-galactosyltransferase. This strain was also able to express the *lacY* gene for lactose uptake.
The method caused high formations of acceptor substrates lacto-N-neotetraose and lacto-N-neohexaose with a concentration higher than 5 g / L [84].
It was shown that the acceptor substrate lacto-N-neotetraose can be converted to different fucosylated HMO using various fucosyltransferases, especially those of *Helicobacter pylori* since neither of the two mammalian genes *fut1* and *fut2* have been reported to be functionally expressed in *Escherichia coli* [15].
Although whole cell systems show a lot of advantages compared to other established methods in HMO production they show also drawbacks:
For instance these systems usually need two phases for successful HMO production, a growth phase followed by the production phase which often causes a low overall production rate and a higher conversion of the substrate to biomass instead to the desired product(s).
This disadvantage can be circumvented by developing a whole cell system which requires just one carbohydrate resource and contains a production pathway and a growth (biomass) pathway. This system probably enables a more efficient production of human milk oligosacharides such as 2`-fucosyllactose or 3`-fucosyllactose as well as other compounds [9].
In the past, several experiments with transgenic mammals were performed that resulted in the successful expression and in vivo synthesis of desired primary gene products that are enzymes such as fucosyltransferases [42].
New researches reveal the possibility to work with transgenic animals to produce so called secondary transgenic products like glycolipids, glycoproteins and glycooligosaccharides such as HMO that result from the activity of primary gene products respectively fucosyltransferases.
These laboratory animals are adapted for HMO production since they show very simple milk oligosaccharide profiles which facilitates detection and quantization of (neo-)oligosaccharides.
Experiments with transgenic rabbits and mice have already demonstrated that production of 2`-fucosyllactose is principally feasible [12;35].
Isolated and purified human glycooligosaccharides like fucosylated HMO can be added to infant formulas or used as epitopes to prevent pathogen infections.

Prieto et al. [35;42] could demonstrate that expression of such secondary gene products in mammary glands of transgenic animals is possible which shows the importance of transgenic animals as a kind of "bioreactor".
Transgenic animals that represent a "bioreactor" facilitate the synthesis of human milk oligosaccharides (HMO) in different ways.
For instance catalytic amounts of specific glycosyltransferases like fucosyltransferases are sufficient to produce HMO.
Further on the origin of the transgene might not be essential as long as the efficient production of HMO can be obtained.
Last but not least some transgenic animals such as mice show glycosyltransferases homologous to human ones which enables the production of human oligosaccharides through homologous production in these transgenic animals [12].
In future, transgenic cows could be highly suitable for the production of oligosaccharides since a single cow can produce more than 10.000 liters of milk.
Further metabolic engineering seems to be important since bovine milk mainly contains lactose and misses numerous natural occurring milk oligosaccharides homologous to human milk oligosaccharides (HMO) [12].
To sum up, many factors play an important role in efficient metabolic engineering and in vivo synthesis of different fucosylated HMO such as relative availabilities of substrates, donors and cofactors or the redox status within the cell as well as the choice of host and enzyme(s) from other origin(s) [10].
The following sections precisely describe in vivo production systems used for the synthesis of 2`-fucosyllactose, GDP-L-fucose as well as fucosylated, more complex human milk oligosaccharids (HMO) in bacteria respectively transgenic mammals.

In vivo synthesis of 2`-fucosyllactose in Escherichia coli strains by fucosyltransferase FucT2 (FutC) of Helicobacter pylori

Different in vivo production systems are used in practice with metabolically engineered *Escherichia coli* cells as preferred host strains.
One of these systems is based on two metabolic pathways: The de novo synthesis pathway and the salvage synthesis pathway that are both applicable in the production of GDP-L-fucose, an intermediate in the synthesis of 2`-fucosyllactose [13].
In figure 1 those pathways and the essential transfer reaction of GDP-L-fucose to lactose by fucosyltransferase, in this case fucosyltransferase Fut C or synonymic FucT2 from *Helicobacter pylori* as well as the uptake of required carbohydrate resources by transport proteins are depicted schematically.
Fucosyltransferase FucT2 from *Helicobacter pylori* is the first bacterial α1,2-FucT biochemically characterized and often used for studies in 2`-fucosyllactose production [13;81] [9;75] although it must be mentioned that other fucosyltransferases from alternative bacterial origins have been already examined such as α1,2-FucT of *E. coli* O128:B12 which is also probable to synthesise 2`-fucosyllactose and other important fucosylated glycoconjugates [28].
As seen in figure 9, one opportunity for the synthesis of 2`-fucosyllactose is the uptake of L-fucose by fucose permease, which is then converted into GDP-L-fucose by the salvage GDP-L-fucose pathway enzyme FKP. FKP requires ATP and GTP as cofactors that come from Central Metabolism in *Escherichia coli.*

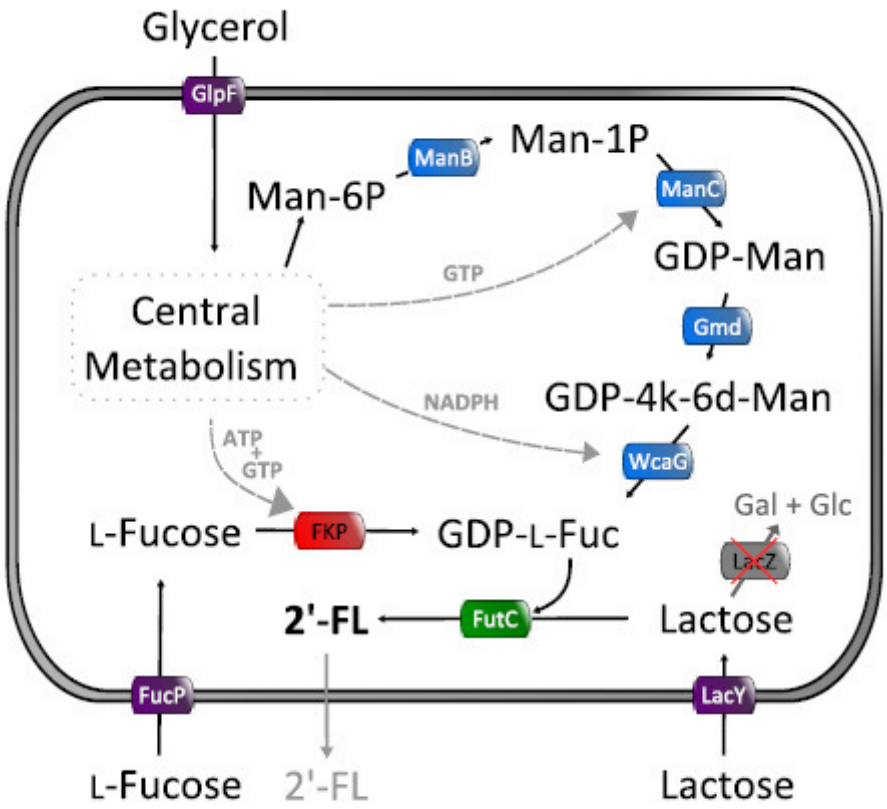


Figure 9 Metabolic pathways I for the in vivo synthesis of 2`-fucosyllactose (2`-FL) in *Escherichia coli* adapted from Baumgärtner et al. [13]. Red, Salvage synthesis pathway. Blue: De novo synthesis pathway. Green, Fucosyltransferase FutC. Molecules and enzymes are abbreviated as follows: Gal, D-galactose. GDP-4k-6d-Man, GDP-4keto-6-deoxymannose. GDP-Man, GDP-α-D-mannose. GDP-L-Fuc, GDP-β-L-fucose. Glc, D-glucose. FucP, fucose permease. GlpF, glycerol MIP channel. LacY, lactose permease. Man-1P, α-D-mannose-1-phosphate. Man-6-P, α-D-mannose-6-phosphate. ManB, phosphomannomutase. ManC, mannose-1-phosphate guanylyltransferase. Gmd, GDP-mannose 4,6 dehydratase. WcaG, GDP fucose synthase. FutC, α1,2-fucosyltransferase. LacZ, β-galactosidase

For Central Metabolism an alternative carbohydrate resource to L-fucose and lactose such as glycerol is taken up by the cell by the transport protein glycerol MIP channel.
Glycerol is then converted to Man-6-P by enzymes of the Central Metabolism, which provides cofactors ATP, GTP and NADPH for further reactions concerning the de novo or salvage synthesis pathway. Therefore an alternative carbohydrate like glycerol or mannose serves as C- and energy source for the de novo synthesis and / or salvage synthesis pathway in the Central Metabolism [13;81].

It is also possible to use lactose as C- and energy source and additional substrate for 2`-fucosyllactose production but yields of 2`-fucosyllactose might be significantly smaller. Lee et al. [81] could demonstrate that more than 90 % of lactose as only carbohydrate resource was metabolized in a batch process for other products such as biomass and endogenous products than for GDP-L-fucose and 2`-fucosyllactose.
Further metabolically engineering might be important to achieve higher product formation from one carbohydrate resource which will be explained later on.
Man-6-P is the end product of Central Metabolism and the substrate of the de novo synthesis pathway. It is converted to GDP-L-fucose by a reaction cascade of the de novo synthesis pathway including the enzymes phosphomannomutase ManB, mannose-1-phosphate guanyltransferase Man C, GDP-mannose 4,6-dehydratase Gmd and GDP-fucose synthase WcaG [13].
GDP-L-fucose and lactose, that were taken up through fucose permease FucP and lactose permease LacY, are converted into 2`-fucosyllactose by an α1,2-fucosyltransferase such as fucosyltransferase FutC of *Helicobacter pylori* [13].
To avoid the consumption of lactose to galactose and glucose by the enzyme betagalactosidase LacZ, the cell as an efficient in vivo production system for 2`-fucosyllactose should be deficient in betagalactosidase LacZ activity as illustrated in figure 1 [13].

In vivo synthesis of 2`-fucosyllactose by genomic integration of gene *futC* of Helicobacter pylori

Different metabolic engineered cell expression systems with *Escherichia coli* JM109 as host strain were reported by Baumgärtner et al. [13] that are based on the metabolic pathways of figure 1 and harbouring genes for the de novo and / or the salvage synthesis pathway as well as the fucosyltransferase gene *futC*.
Escherichia coli JM109 is a permease positive but betagalactosidase LacZ negative strain due to deletions in both genomic and episomal copies of the *lacZ* gene which allows uptake of lactose and its conversion into 2`-fucosyllactose instead of galactose and glucose.
In this study different selection marker free *Escherichia coli* strains JM109 that produce 2`-fucosyllactose from lactose as substrate for fucosylation and glycerol as carbon- and energy

source were constructed by genomic integration of expression cassettes and knocking out certain sugar degradation genes using the Lambda-Red recombineering technique to avoid the transformation of plasmids and use of antibiotics for selection [13;68].

Plasmids can also be transformed and used as strategy to produce 2`-fucosyllactose but are often structural and segregational instable. As a reason a selection marker like an antibiotic resistance gene is needed for avoiding a plasmid loss in the cell but the use of antibiotics is often undesirable, especially in food industry.

Moreover a metabolic burden effect can be caused by an unequal expression of genes needed for synthesis of 2`-fucosyllactose because of a different amount of plasmid copies and / or promoter strength in the cell that can lower the biomass and / or product yield [13].

As an alternative, genetic modifications in *Escherichia coli* strains JM109 were implemented by Baumgärtner et al. [13] by using expression cassettes as can be seen in figure 10:

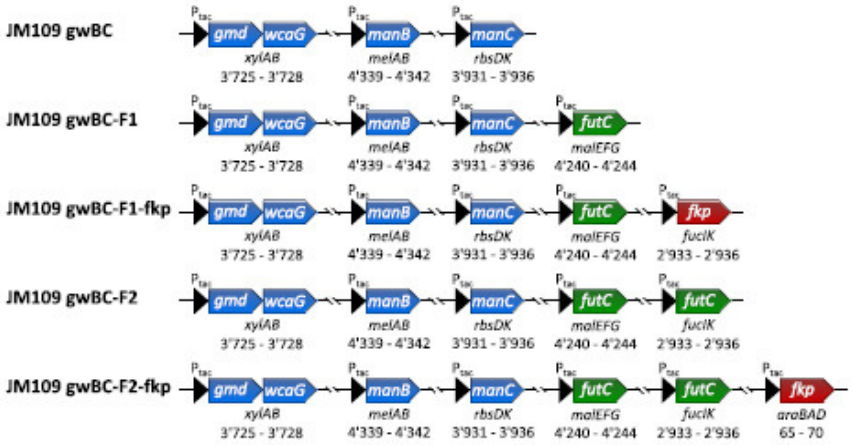


Figure 10 Expression cassettes integrated in *Escherichia coli* adapted from Baumgärtner et al. [13]. Abbreviations: *gmd*, GDP-D-mannose dehydratase gene. *wcaG*, GDP fucose synthase gene. *manB*, phosphomannomutase. *manC*, mannose-1-phosphate guanylyltransferase. *futC* α1,2-fucosyltransferase gene. *fkp*, salvage synthesis pathway enzyme. Arrows symbolize tac promoters (P_{tac}). Knocked out by knocked in area in kb and degradation genes are given below integration cassettes

Genes for fucosyltransfrase FutC from *Helicobacter pylori* and the de novo synthesis pathway and salvage pathway from different bacterial origins are depicted and described in figure 11. They were integrated as individual expression cassettes with an IPTG-inducible P_{tac}-promotor into the genome of *Escherichia coli* strain JM109 [13].

Lee et al. [82] demonstrated that amplification of genes of the de novo synthesis that are essential for the synthesis of GDP-mannose is substantial in an efficient production of GDP-fucose: In a glucose-limited fed-batch process it was observed that introduction of genes *gmd* and *wcaG* had a positive influence on synthesis but that integration of genes *manB* and *manC* in combination with *gmd* and *wcaG* showed a more than 4 times better result in production. Gene *manA* was not integrated and overexpressed. According to Lee et al. [82] it should not show any positive influence on GDP-fucose production and thus probably has no effect on 2`-fucosyllactose synthesis too.

Different *Escherichia coli* strains were constructed by Baumgartner et al. [13] that all contain the expression cassettes for the de novo synthesis pathway depicted in figure 10:

JM109 gwBC with integrated expression cassettes for the de novo synthesis pathway only, JM109 gwBC-F1 with an additional expression cassette for fucosyltransferase FutC activity, JM109 gwBC-F1-fkp with an expression cassette for fucosyltransferase FutC activity and an additional *fkp* expression cassette for the salvage synthesis pathway, JM109 gwBC-F2 with two *futC* expression cassettes for fucosyltransferase FutC activity and JM109 gwBC-F2-fkp with two *futC* expression cassettes for fucosyltransferase FutC activity and the *fkp* expression cassette for the salvage synthesis pathway.

Degradation of intracellular fucose was prevented by knocking out the genes *fucK* for fuculose-kinase and for fucose-isomerase through insertion of *fkp* or a second *futC* gene.

Comparisons between 2`-fucosyllactose and intracellular GDP-L-fucose yields of the different strains that were obtained via batch processes in shake flask experiments showed that mainly a further salvage synthesis pathway beside the de novo synthesis pathway enabled higher 2`-fucosyllactose and GDP- L-fucose yields within the cell [82].

Also a second copy of the *futC* gene had a positive, but smaller effect on 2`-fucosyllactose yield. Therefore *Escherichia coli* strain JM109 gwBC-F2-fkp showed the highest 2`-fucosyllactose yield of nearly 400 mg g_{CDW}^{-1} closely followed by strain JM109 gwBC-F1-fkp with just one copy of futC [82].

Yields of strains JM109 gwBC-F1 and JM109 gwBC-F2 were also in the same order and significantly smaller as those of strains JM109 gwBC-F2-fkp and JM109 gwBC-F1-fkp as can be seen in figure 11 and 12 adapted from Lee et al. [82]:

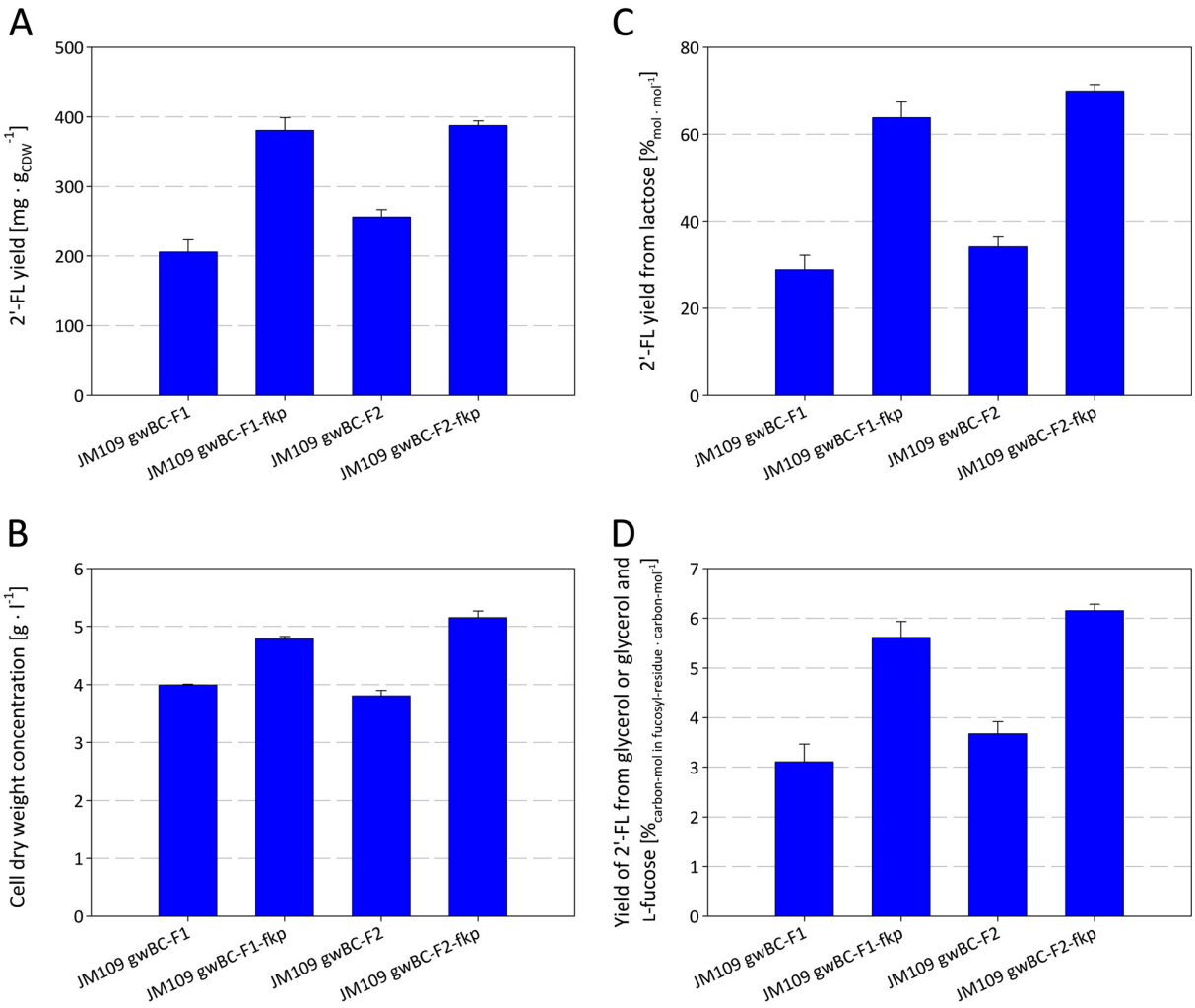


Figure 11 Comparison of modified *Escherichia coli* strains in shake flask experiments adapted from Lee et al. [82]. A) 2′-fucosyllactose (2`-FL) yield of batch cultivated strains with or without salvage GDP-L-fucose production 24 hours after induction. B) Cell dry weight concentrations corresponding to Figure 5A.
C) Produced 2′-FL from lactose corresponding to Figure 5A. D) Percentage of carbon atoms converted from glycerol or glycerol and L-fucose to the fucosyl-residue of the produced 2′-FL corresponding to Figure 5A

Strain JM109 gwBC-F2 with the de novo synthesis pathway only produced 30 % less 2`-fucosyllactose compared to strain JM109 gwBC-F2-fkp with additional salvage synthesis pathway but only 15% more than strain JM109 gwBC-F1 with just one copy of *futC*.
Figure 3 also shows that the biomass yield (Cell dry weight concentration) increased significantly in strains JM109 gwBC-F1-fkp and JM109 gwBC-F2-fkp through incorporating a further salvage synthesis pathway which leads to the conclusion that the added L-fucose for

salvage synthesis pathway is mainly involved in the synthesis of GDP-L-fucose and 2`-fucosyllactose than in anabolic reactions [82].

The biomass yield of strains containing the *fkp* gene and thus produce GDP-L-fucose mainly from L-fucose based salvage synthesis pathway increased by 20% compared to those strains that only contained the de novo synthesis pathway [82].

This could mean that the subtraction of NADPH and fructose-6-phosphate by an only de novo synthesis pathway containing strain causes a decrease in the energy and carbon pool within the cell which in turn lowers the biomass yield.

In figure 12 the intracellular concentrations of GDP-L-fucose in *Escherichia coli* strains with or without salvage synthesis pathway for GDP-l-fucose production after induction through IPTG are depicted [82]:

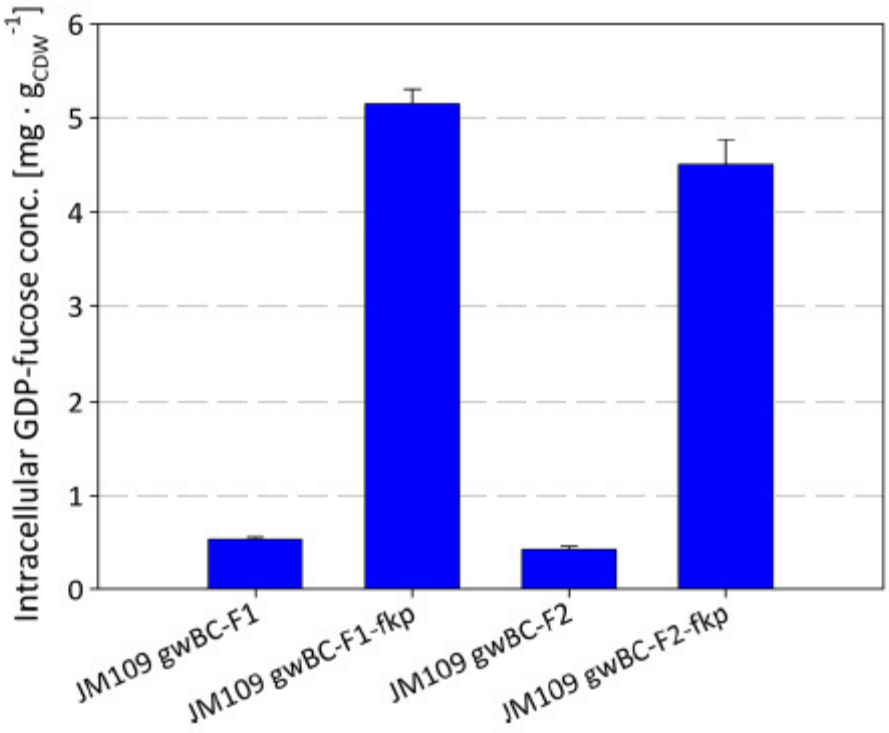


Figure 12 Intracellular concentrations of GDP-L-fucose in *Escherichia coli* strains without or with salvage synthesis pathway for GDP-L-fucose production 12 hours after induction adapted from Lee et al. [82]

The incorporated salvage synthesis pathway in a de novo synthesis pathway containing strain could improve the intracellular GDP-L-fucose yield about 10 times whereas a second *fut C* gene had a smaller influence on it.

In contrast to the 2`-fucosyllactose yield, where JM109 gwBC-F2-fkp showed a higher 2`-fucosyllactose yield than JM109 gwBC-F1-fkp, a higher GDP-L-fucose yield could be

reached in JM109 gwBC-F1-fkp of up to 5,1 mg g_{CDW}^{-1} whereas for JM109 gwBC-F2-fkp a smaller GDP-L-fucose yield of about 4,5 mg g_{CDW}-1 was measured [82].
A second *fut C* gene of JM109 gwBC-F2-fkp could have lead to a stronger expression level of fucosyltransferase gene *futC* and thus to a stronger conversion of GDP-L-fucose to 2`-fucosyllactose and a smaller GDP-L-fucose -respectively higher 2`-fucosyllactose -yield.
As a result in JM109 gwBC-F2-fkp the highest 2`-fucosyllactose yield of 387,76 ± 6,71 mg / g_{CDW} could be determined [82].
All *Escherichia coli* strains used for batch fermentations were cultivated in shake flasks and fed with glycerol. Strains JM109 gwBC-F1-fkp and JM109 gwBC-F2-fkp were additionally provided with L-fucose.
Although *Escherichia coli* JM109 gwBC-F2-fkp showed the highest 2`-fucosyllactose yield and concentration, JM109 gwBC-F2 was used to demonstrate an antibiotic- free fed- batch fermentation in a stirred-tank reactor with a fed batch cultivation containing a batch volume of 8,4 L and a total feed volume of 5,1 L using mineral salt medium.
A significant higher amount and concentration of 2`-fucosyllactose of 273 g respectively 20,28 ± 0,83 g / L culture volume was reached by a final cell dry weight concentration of 65,61 g / L which approximately correlates to the shake flask experiment where a total amount of about 24 mg 2`-fucosyllactose and a 2`-fucosyllactose concentration of approximately 0,968 g / L culture volume by a final cell dry weight concentration of approximately 3,75 g / L was observed [82].
Furthermore a 2`-fucosyllactose yield of 0,309 ± 0,013 g / $g_{CDW}$ was obtained which is higher than the yield of *Escherichia coli* JM109 gwBC-F2 used for batch fermentation in the shake flask experiment with approximately 0,256 g 2`-fucosyllactose / g CDW [82].

In vivo synthesis of 2`-fucosyllactose without genomic integration of *futC* of Helicobacter pylori

In a previous approach Lee et al. [81] could produce 2`-fucosyllactose through a whole cell biosynthesis system containing *Escherichia coli* strain JM109 (DE3) by the transformation of two plasmids without genomic cassette integration:
Plasmid pHfucT2 which carries the gene for fucosyltransferase FutT2 and plasmid pmBCGW which includes a polycistronic gene cluster *gmd-wcaG* and a gene cluster *manB-manC*.

The whole cell approach was based on the de novo synthesis pathway depicted in figure 1 [13] and the expression of a single copy of the *futT2* respectively *futC* gene. The salvage synthesis pathway [13] was not incorporated.
Two alternative approaches were tested to measure 2`-fucosyllactose and GDP-L-fucose yields:
One whole cell system which was provided with lactose as only carbohydrate resource and another one with lactose as substrate for 2`-fucosyllactose synthesis and mannose as carbon and energy source. About 0,4 g / L lactose were not consumed within the cell which suggested that at least 0,5 g / L lactose should be added to the cell to observe 2`-fucosyllactose production.
The whole cell system which was provided with lactose only showed a yield of 0,06 ± 0,005 g / g lactose, those with lactose as substrate for 2`-fucosyllactose synthesis and mannose as carbon and energy source a weak 2`-fucosyllactose yield increase of 0,13 g / g lactose which led to the conclusion that in both approaches only a small amount of lactose was consumed for 2`-fucosyllactose production [81].
Compared to *Escherichia coli* strain JM109 gwBC – F1 by Baumgartner et al. [13] with also one copy of the *fut C* gene and a yield of 2`-fucosyllactose of approximately 0,409 g / g lactose, the yield of both whole cell systems by Lee et al.[81] was about 3-7 times lower.
One explanation for the big differences in yields concerning strain JM109 gwBC – F1 with lactose and glycerol as carbohydrate resource and strains JM109 (DE3) provided with lactose and mannose as carbohydrate resource could be that glycerol can be better converted in Central Metabolism than mannose and thus more lactose is avalaible for 2`-fucosyllactose synthesis although it must be included that for JM109 gwBC – F1 the concentration of glycerol was twice as high as the concentration of mannose for JM109 (DE3).
It is also possible that the integration of expression cassettes is more efficient in production of 2`-fucosyllactose because a plasmid loss within the cell could have been avoided.
For Lee et al. [81] it is conceivable that insertion of the Lambda(DE3) cassette end expression of it to a functional α-peptide caused a so called α complementation and thus functionality of the *lacZ* gene. Therefore a not incompletely inactivated betagalactosidase could have been expressed by α complementation which could have reduced 2`-fucosyllactose yield [16;81;85].
To obtain higher yields of 2`-fucosyllactose a batch cultivation was performed by Lee et al. [81] with a higher lactose concentration of about 15 g / L and without adding mannose.

A final 2`-fucosyllactose concentration of 1,23 ± 0,011g 2`-fucosyllactose / L final culture volume respectively a yield of 0,09 ± 0,004 g 2`-fucosyllactose / g lactose was determined [81].
Although it is principally known that *Escherichia coli* synthesises acetate by higher concentrations of sugar resources it could be demonstrated that there was no production of it when a slow consumption of lactose takes place. This facilitates operation within a fed batch reactor [81;82].

Enhancement of GDP-L-fucose formation in order to produce significant higher amounts of 2`-fucosyllactose

Since it is known that NADPH is widely consumed in biomass and metabolite production such us xylitol [82] it is useful to increase the energy pool in a de novo and / or salvage synthesis pathway harbouring strain by overexpression of NADPH generating enzymes of Central Metabolism because enzyme GDP-fucose-synthase needs NADPH as cofactor and therefore sufficient amounts of NADPH play a key role in GDP-L-fucose production [81]. Also a higher availability of other cofactors like ATP or GTP might have a positive effect on GDP-L-fucose content.
NADPH can be regenerated in Central Metabolism by different prokaryotic enzymes such as isocitrate dehydrogenase (Icd) in the tricarboxylic acid (TCA) cycle, glucose 6-phosphate dehydrogenase (G6PDH) and 6- phosphogluconate dehydrogenase (Gnd) in the oxidative pentose phosphate pathway (PPP), pyridine nucleotide transhydrogenases (soluble transhydrogenase UdhA and membrane-bound transhydrogenase PntAB) and NADP+-dependent malate dehydrogenase (MaeB) in the anaplerotic pathway [82].
TCA cycle and PPP may represent the primary pathways in NADPH-generation [86] since 35–45% and 20–25% of the NADPH is produced by PPP and TCA (Icd), respectively
But according to a study by Sauer et al. [86] also a high percentage of NADPH of 35–45% can be produced via the proton-translocating and membrane-bound transhydrogenase PntAB during standard aerobic batch growth on glucose.
NADPH is needed as cofactor for anabolic reactions such as cell growth in general or particularly for synthesis of GDP-L-fucose, which is an intermediate in the production of 2`-fucosyllactose [82;86].

There exist two transhydrogenase isoforms that occur mainly separated in different prokaryotes and show divergent physiological functions: Energy-dependent reduction of $NADP^+$ with NADH by the mentioned transhydrogenase PntAB and reoxidation of NADPH by the soluble transhydrogenase UdhA.
Both could be functionally expressed in *Escherichia coli* MG1655 by Sauer et al. [86].
They create a flexibility to cope with imbalances between catabolic NADPH production and anabolic NADPH consumption in Escherichia coli
More precisely the expression patterns of the *udhA* gene encoding the soluble transhydrogenase UdhA and the *pntA* gene encoding the membrane-bound transhydrogenase PntAB indicates transcriptional regulation that responds to the redox state of cellular metabolism as could be demonstrated in the study by Sauer et al. [86].
A dramatic excess of NADPH formation could be obtained in a phosphoglucose isomerase deficient mutant of *Escherichia coli* MG1655 called Pgi through increased PP pathway fluxes and an interrupted glycolysis because of a deficient phosphoglucose isomerise [86].
Interestingly a reduced *pntA* transcription was observed in this Pgi mutant and might reveal that the NADPH formation by PntAB was apparently not essential for growth on glucose under metabolic conditions with excess NADPH formation.
In contrast to PntAB, UdhA was identified to be essential for producing NADP under conditions with an excess of NADPH formation since transcription of gene *udhA* was not down-regulated.
This observation supports the notion that the physiological role of UdhA is to convert excess NADPH into NADH which can enter the respiratory chain for energy generation.
Analogously, *udhA* transcription in an *Escherichia coli* wild type on glycerol was down-regulated when NADPH formation was reduced in order to produce more NADPH whereas transcription of PntAB was unchanged. Down-regulation on glycerol was observed since growth on glycerol required a higher formation of NADPH than on glucose.
Because expression of genes *pntA* and *udhA* may respond conversely to genetic or environmental dependent availabilities of NADPH, a NADPH-dependent repression mechanism may be behind it [86].
Expression of PntAB could lead to an improvement of GDP-fucose synthesis in *Escherichia coli* because usually no excessive amounts of NADPH as for instance through an enhanced flux to PP are present within the cell [86].
So far, *Escherichia coli* does not have a native enzyme catalyzing direct conversion of NADH to NADPH and has only NAD kinase that is known to convert NAD to NADP [80]. Thus it

requires enzymes such as PntAB for the transhydrogenase reaction of NADH and NADP to NAD and NADPH since NAD is essentially entirely -linked to the catabolism and NADPH to anabolic reactions [87] such as GDP-L-fucose synthesis.
In figure 13 reactions and possible enzymes that can be used for NADPH generation and consumption as well as anabolic GDP-L-fucose formation in *Escherichia coli* are shown [82]:

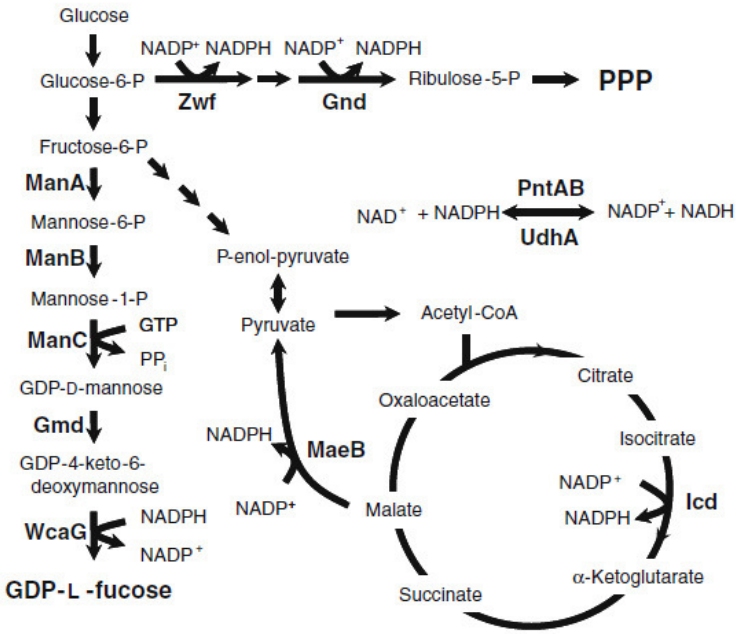


Figure 13 Metabolic pathway of GDP-fucose synthesis and NADPH regeneration within *Escherichia coli* adapted from Lee et al. [82]. Abbreviatons: ManA, ManB, phosphomannomutase. manC, mannose-1-phosphate guanylyltransferase. Gmd, GDP-D-mannose dehydratase. WcaG, GDP fucose synthase.. Zwf, glucose-6-phosphate 1-dehydrogenase. Gnd, 6-phosphogluconate dehydrogenase. PntAB, UdhA, transhydrogenases

Lee et al. [82] could prove that GDP-L-fucose synthesis in *Escherichia coli* TOP10 strains could be increased by overexpression of genes for endogenous NADPH regenerating enzymes isocitrate dehydrogenase (Icd), glucose-6-phosphate dehydrogenase (G6PDH) and NADP+-dependent malate dehydrogenase (MaeB) whereby glucose-6-phosphate dehydrogenase (G6PDH) in combination with a glucose feeding strategy in a fed batch process had the best impact on GDP-L-fucose production.
First four *Escherichia coli* strains were constructed and used in batch processes to express the essential genes for GDP-L-fucose production:

A control strain overexpressing de novo synthesis pathway genes *manB, manC, gmd* and *wcaG*, a *zwf* overexpressing strain, an *icd* overexpressing strain and a *maeB* overexpressing strain.

Among the four strains, the *zwf* overexpressing strain gave the best results: 6.4± 0.24 mg / L of GDP-L-fucose were produced and resulted in a 46% increase compared to the control strain [82].

GDP-L-fucose concentration could also be improved by 31% when *icd* was overexpressed.

Only the *maeB* overexpressing strain showed with a GDP-L-fucose concentration of 3.3±0.04 mg / L a lower concentration of 24% than the control strain.

Gene *maeB* was not convenient for a higher GDP-L-fucose production probably because of a weaker metabolic flux from the anaplerotic pathway compared with other pathways like PPP and TCA [82].

From the study by Lee et al. [82] it is obvious that a seperated overexpression of NADPH regenerating enzymes of the Central Metabolism has a stronger influence on higher GDP-6-fucose concentrations than overexpression of the de novo synthesis enzymes since G6PDH was tested as the most effective enzyme in GDP-L-fucose production followed by Icd.

The highest concentration of GDP-L-fucose could be reached with an *Escherichia coli* BL21(DE3) star strain through simultaneous overexpression of genes for glucose-6-phosphate dehydrogenase (G6PDH) and enzymes ManB, ManC, Gmd, and WcaG in a pH-stat feeding mode based fed batch process.

235.2 ± 3.3 mg / L were measured and yielded an enhancement of GDP-L-fucose concentration of 21 % compared to a *manB, manC, gmd*, and *wcaG* overexpressing control strain in a pH-stat feeding mode based fed batch process [82].

To overexpress GDP-L-fucose, an earlier strategy was implemented by Lee et al. [88] first in which plasmid pmBCGW containing the polycistronic *gmd–wcaG* and *manB–manC* gene clusters and coding for ManB, ManC, Gmd, and WcaG was transformed.

In a posterior second approach by Lee et al. [82] plasmid pmBCGW as well as plasmid pMWzwf including the *zwf* gene for G6PDH were transformed.

Both were used for fed batch fermentations which are illustrated in table 7 [82]:

Table 7 Fed batch fermentations of *Escherichia coli* BL 21star(DE3) strains adapted from Lee et al. [82]

Plasmid	Overall glucose feed rate[a] ($g\ h^{-1}$)	Dry cell mass ($g\ l^{-1}$)	GDP-L-fucose concentration ($mg\ l^{-1}$)	GDP-L-fucose productivity[b] ($mg\ l^{-1}\ h^{-1}$)	Specific GDP-L-fucose content ($mg\ g cell^{-1}$)	Yield[c] (mg GDP-L-fucose g consumed glucose^{-1})
pmBCGW[c]	7.0	74.2±0.5	170.3±2.3	6.7±0.09	2.3±0.03	1.2±0.02
pmBCGW+pMWzwf	7.0	75.3±0.6	141.8±1.9	5.8±0.08	1.9±0.03	1.1±0.01
pmBCGW	15.0	85.0±0.6	193.6±1.9	10.2±0.10	2.3±0.02	1.0±0.01
pmBCGW+pMWzwf	14.1	84.3±0.8	235.2±3.3	12.7±0.18	2.8±0.04	1.3±0.02

When complete depletion of 2% (w/v) glucose added initially was reached, a concentrated solution consisting of glucose and MgSO4·7H2O was fed by constant feeding (rate, 7.0 g / h) or pH-stat feeding (overall rate, ≥14.0 g / h) mode. The concentrated solution was added automatically to the bioreactor when glucose was depleted for pH-stat feeding.

In contrast to the batch fermentations, no improvement in GDP-L-fucose synthesis could be obtained in a fed-batch fermentation of the *zwf* overexpressing *Escherichia coli* strain with constant feeding of glucose with regard to the same fed-batch fermentation of an *Escherichia coli* control strain [82]. A limited carbon source related to insufficient supplementation of glucose especially in GDP-L-fucose production could be responsible for a reduction of GDP-L-fucose content since the carbon flux from glucose to GDP-L-fucose could have been reduced because of a stronger flow to PPP and a shift of the carbon flux to ribulose-5-phosphate by overexpression of gene *zwf* for NADPH regenerating G6PDH [14]. As shown in Table 7, overexpression of *zwf* and a constant glucose feed rate did not increase specific GDP-fucose content nor yield from glucose but reduced both by 17% respectively 8% compared with the control strain.

A fed batch process with a *zwf* overexpressing *Escherichia coli* strain based on a ph dependent feeding rate of glucose could enhance the yield of GDP-L-fucose by 30% compared to the same fed batch process with an *Escherichia coli* control strain [82]. Efficient utilization of glucose seems to be crucial for NADPH regeneration as well as carbon supply for GDP-L-fucose production according to the obtained GDP-L-fucose yields. Moreover an improvement in GDP-L-fucose production of 21% could be reached by a ph stat feeding rate based fed batch process with glucose and *zwf* overexpressing *Escherichia coli* compared with a constant feeding rate based fed batch process with glucose because of a higher and sufficient amount of glucose [82].

Beside an increase in GDP-L-fucose yield acetate formation could be reduced by 75% through overexpression of *zwf* and efficient regeneration of NADPH in the *zwf* overexpressing strain with regard to the control strain [82].
Acetate formation based inhibitory effects such as reduction of cell growth rate and inhibition of nucleotide and protein biosynthesis could not be observed in the pH-stat fed-batch fermentation of the *zwf* overexpressing strain in contrast to those of the control strain [82].
As shown in Fig. 14 [82], cell growth as well as GDP-L-fucose production rate was reduced in both, control strain and *zwf* overexpressing strain with acetate accumulation in pH-stat feeding based fed batch processes whereby the *zwf* overexpressing strain was able to show a relative high production and growth rate until the end of fed batch fermentation.

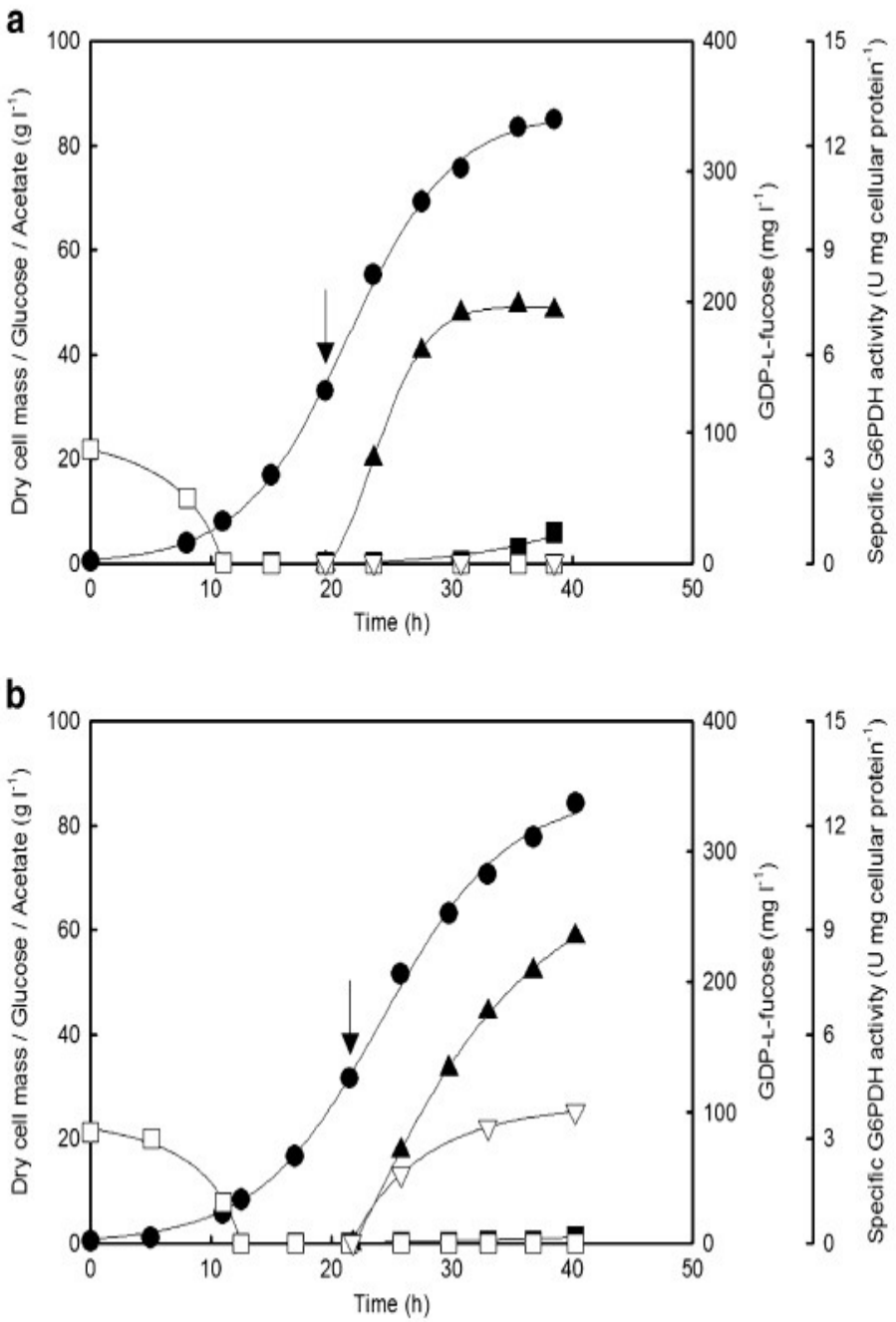


Figure 14 Profiles of GDP-L-fucose synthesis in ph-stat fed-batch fermentations of *Escherichia coli* BL21star(DE3) with plasmid pmBCGW (a) and pmBCGW and pMWzwf(b) adapted from Lee et al. [82]. Arrows represent addition of 0,1 mM IPTG. The data represent average values from three independent measurements. Symbols: Filled triangle, GDP-L-fucose concentration. Filled circle, dry cell mass. Filled square, acetate concentration. Empty inverted triangle, specific Glucose-6-phosphate-dehydrogenase activity

The arrow in figure 14 indicates that IPTG was addicted to the culture which led to a linear increase of GDP-L-fucose. This increase could be to a greater or lesser extent maintained till the end of fermentation because acetate formation (1.4 g / L) was reduced more than 4 times compared to the control strain. G6PDH activity was maintained higher than 2 U / mg cellular protein in the G6PDH overexpressing strain [82].

As shown in Table 8, NADP+ and NADPH levels of a *zwf* overexpressing strain were elevated by 20% and 65%, respectively, whereas NAD+ and NADH levels were reduced by 43% and 14%, compared to a control strain which only contained the empty plasmid pACYCDuet-1. NADP+ and NADPH levels were measured from a whole cell system of *Escherichia coli* with the same medium conditions as in the fed batch processes [82].

Table 8 Cofactor concentrations and ratios measured in G6PDH overexpressing Escherichia coli strain and *Escherichia coli* control strain adapted from Lee et al. [82]

Plasmid	NAD^+ (µmol/gcell)	NADH (µmol/gcell)	NADP (µmol/gcell)	NADPH (µmol/gcell)	Ratio of $NADH/NAD^+$	Ratio of $NADPH/NADP^+$	Total concentrations (µmol/gcell)
pACYCDuet-1	2,91±0,05	0,70±0,16	1,72±0,06	1,41±0,05	0,24	0,82	6,74
pMWzwf	1,66±0,07	0,60±0,07	2,08±0,01	2,33±0,03	0,36	1,12	6,68

The ratios NADH/NAD+ and NADPH/NADP+ in the *zwf* overexpressing strain were higher than the control strain even through reduction of NAD+ and NADH levels. Moreover it could be observed that the sum of NAD+, NADH, NADP+, and NADPH concentrations in the *zwf* overexpressing strain was almost the same as those in the control strain [82]. These results indicate that the total level of pyridine nucleotides is constant but effective regeneration of NADPH can be achieved by *zwf* overexpression in a recombinant *Escherichia coli* strain [7].

Beside different experiments in order to improve GDP-L-fucose concentrations and to obtain higher 2`fucosyllactose yields it must also be considered to reduce the consumption of GDP-L-fucose to other cellular products than 2`fucosyllactose such as colonic acid, an important component in bacterial cell wall synthesis in *Escherichia coli*.

Since GDP-L-fucose is a precursor of colonic acid in *Escherichia coli* one opportunity to avoid its consumption to it is to simultaneously overexpress the positive regulatory protein and transcriptional activator RcsA of the colonic acid operon beside gene *futC* and to knock out genes involved in the colonic acid synthesis such as *wcaJ* [81-83].

Alternatively or additionally to the mentioned enzyme candidates that can be used for NADPH regeneration in GDP-L-fucose- or 2`-fucosyllactose production, direct conversion of NADH to NADPH through direct phosphorylation could be a further and even more efficient tool to increase the intracellular NADPH availability and productivity in biotransformation processes.

Since high amounts of NADH can be generated in glycolysis from *Escherichia coli* cells a direct conversion could yield high concentrations of NADPH within the organism.

Because *Escherichia coli* does not contain such enzymes, a gene for NADH kinase called *pos5p* from *S. cerevisiae* with a more than 50 times higher specificity to NADH than NAD was overexpressed in fed-batch fermentations in *Escherichia coli* BL21star(DE3) in a previous study by Lee e al. [80].

A fed-batch fermentation of recombinant *E. coli* BL21star(DE3) harbouring plasmid pmBCGW and pHpos5 with the gene *pos5p* for NADH kinase activity was performed. This fed batch fermentation was compared to fed batch fermentations of a control strain harbouring the plasmid pmBCGW only and a G6PDH overexpressing strain harbouring the plasmid pmBCGW and pMWzwf with the gene for G6PDH activity.

NADP+ and NADPH levels of the *pos5p* expressing strain were increased by 13 % and 33 %; respectively, whereas NAD+ and NADH levels could be lowered by 21 % and 20 %, respectively, compared with the control strain [80].

It could be obtained that *pos5p* was functionally expressed and effective generation of NADPH was achieved because the ratio of NADH/NAD+ remained stable, whereas the ratio of NADPH/NADP+ increased by 17 % in the *pos5p* expressing strain. Moreover the sum of NAD+, NADH, NADP+, and NADPH concentrations in the *pos5p* expressing strain was almost the same as the sum in the control strain [80].

The rise in NADPH/NADP ratio and the higher specificity for NADH than NAD by enzyme Pos5p might be ascribed to the consumption of NAD+ for complementing lack of NADH caused by *pos5p* expression [80].

.In contrast to expression of genes coding for enzymes such as G6PDH, expression of genes coding for NADH kinase may have an advantage in saving carbon molecules for a more efficient glucose based GDP-L-fucose production which goes well together with the observation by Lee et al. [80] that the *pos5p* expressing strain showed better GDP-L-fucose production and specific GDP-L-fucose content than the control and G6PDH overexpressing strain:

A hyperbolic increase in GDP-L-fucose production of the *pos5p* harbouring strain was observed after addition of IPTG to the cell culture.
In contrast to the control strain, both GDP-L-fucose and cell growth were maintained till the end of fermentation. After 21 h from induction, 291.5 mg/l of GDP-L-fucose concentration and a specific GDP-L-fucose content of 3.1 mg GDP-L-fucose / g cell were measured which corresponded to 51 % respectively 35 % enhancement compared to the control strain [80].
Figure 15 gives an overview about GDP-L-fucose concentration, growth (cell dry mass), glucose consumption and acetate formation observed in the *pos5p* overexpressing strain during fed batch fermentation:

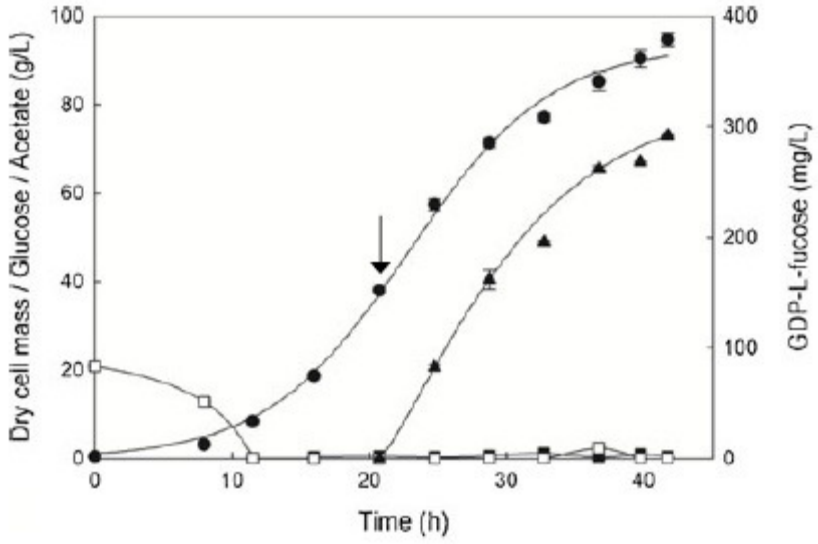


Figure 15 GDP-L-fucose synthesis profile of fed batch fermentation with *Escherichia coli* BL21star(DE3) harbouring pHpos5 and pmBCGW adapted from Lee et al. [80]. Arrow symbolizes addition of 0,1 mM IPTG. Closed circle represents dry cell mass, closed triangle stands for GDP-L-Fucose concentration, open square for glucose concentration and closed square for acetate concentration

The product yield of GDP-L-fucose was improved by 40 % and 8 % compared to the control and G6PDH overexpressing strains, respectively [80].
Formation of acetate which is known to be related with the redox balance of pyridine nucleotides and negative effects on production yields was also significantly lowered in the *Pos5p* expressing strain compared with the other strains. Associated to the enhancement of GDP-L-fucose production, a significant reduction of acetate formation (0.5 g/l) with regard to the control strain (5.9 g/l) was observed [80].

These observations indicate that direct phosphorylation of NADH leads to a faster NADPH regeneration which reduces acetate production within the cell.
Table 9 summarizes and compares the performed fed batch fermentations in the study by Lee et al. [80].

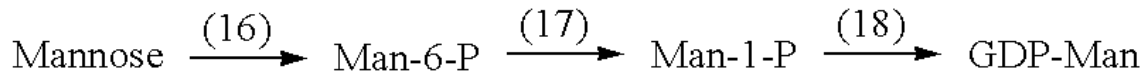
Table 9 Fed- batch fermentations of Escherichia coli BL21star(DE3) adapted from Lee et al. [80]

Plasmids	Dry cell mass (g/l)	GDP-L-fucose concentration (mg/l)	GDP-L-fucose productivity[a] (mg/l·h)	Specific GDP-L-fucose content (mg/g cell)	GDP-L-fucose yield[b] (mg/g consumed glucose)	Acetate concentration (g/l)
pmBCGW[c]	85.0±0.6	193.6±1.9	10.2±0.10	2.3±0.02	1.0±0.01	5.9±0.22
pmBCGW+pMWzwf[d]	84.3±0.8	235.2±3.3	12.7±0.18	2.8±0.04	1.3±0.02	1.4±0.03
pmBCGW+pHpos5	94.7±1.6	291.5±0.8	13.9±0.04	3.1±0.01	1.4±0.01	0.5±0.01

In vivo synthesis of GDP-L-fucose and Lewis X oligosaccharides by bacterial coupling and 1,3-fucosyltransferase of Helicobacter pylori

A large scale production system in order to produce high amounts of GDP-L-fucose, Lewis X and other human milk oligosaccharides (HMO) in an aqueous medium was developed by Koizumi et al. [89;90].
This system is based on two different bacterial strains:
On the one hand recombinant *Escherichia coli* which synthesizes GDP-fucose and the essential fucosyltransferase that is 1,3-fucosyltransferase of *Helicobacter pylori* in the case of Lewis X oligosaccharide synthesis.
On the other hand *Corynebacterium ammoniagenes* producing GTP from GMP.
This in vivo system is based on two production steps:
A GDP-fucose production followed by a Le^x formation.
For GDP-fucose production a fermentation with *Corynebacterium ammoniagenes* and three different metabolic engineered *Escherichia coli* strains NM522 was performed on a 30-mL scale in a 200 mL vessel:

One *Escherichia coli* NM522 strain which overexpressed genes *manB* for phosphomannomutase, *manC* for mannose-1-P-guanylyltransferase, *glk* for glucokinase and *pfk* for phosphofructokinase under the control of a phage lambda promoter.
A second *Escherichia coli* NM522 strain overexpressing gene *gmd* for GDP-mannose dehydratase under the control of a tryptophan promoter.
And a third *Escherichia coli* NM522 strain overexpressing gene *wcaG* for GDP-fucose synthase or synonymous GDP-4-keto-6-deoxy-mannose epimerase / reductase (GMER) under the control of a phage lambda promoter [89;90].
GMP was added for GTP formation by *Corynebacterium ammoniagenes* which might be essential since mannose-1-P-guanylyltransferase is GTP dependent.
Mannose as starting substrate for the conversion to GDP-fucose by NM522 and fructose as starting substrate for glycolysis in order to produce glucose-1,6-bisphosphate by NM522 [89;90].
Formation of glucose-1,6-bisphosphate is thought to be involved in phosphorylation respectively activation of natural occurring phosphoglucosamine mutase GlmM in *Escherichia coli* which is essential for peptidoglycan and lipopolysaccharide biosyntheses and needed for bacterial growth [91].
Both, mannose as well as fructose are converted through separated pathways of NM522 to yield GDP-fucose (pathway 1) and glucose-1,6-bisphosphate (pathway 2), respectively.
A third pathway describes the formation of glucosamine-1-phosphate, an intermediate in peptidoglycan and lipopolysaccharide synthesis by hexokinase respectively glucokinase, and phosphoglucosamine mutase GlmM (pathway 3) [90].
Pathways 1-3 are depicted in figures 16-19 adapted from Koizumi et al. [90]:

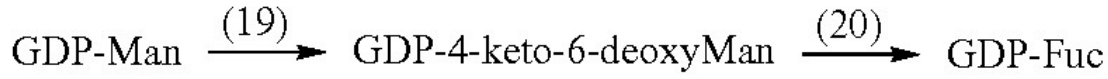


(16): Hexokinase (EC 2.7.1.1) or glucokinase (EC 2.7.1.2)

(17): Phosphomannomutase (EC 2.7.5.7)

(18): Mannose-1-phosphate guanyltransferase (EC 2.7.7.13)

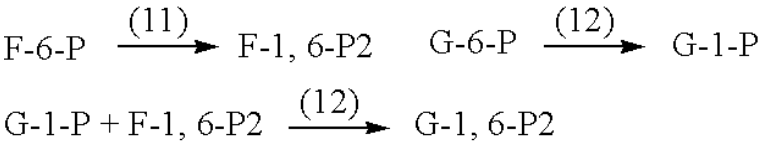
Figure 16 Pathway 1 adapted from Koizumi et al. [90]



(19): GDP-Man-4, 6-dehydratase (EC 4.2.1.47)

(20): GDP-4-keto-6-deoxymannose epimerase/reductase

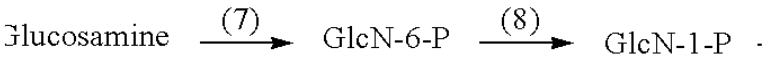
Figure 17 Pathway 1 adapted from Koizumi et al. [90] continued



(11): Phosphofructokinase (EC 2.7.1.11)

(12): Phosphoglucomutase (EC 2.7.5.1)

Figure 18 Pathway 2 adapted from Koizumi et al. [90]



(7): Hexokinase (EC 2.7.1.1) or glucokinase (EC 2.7.1.2)

(8): Phosphoglucosamine mutase

Fifure 19 Pathway 3 adapted from Koizumi et al. [90]

Since GDP-fucose inhibits GDP-man-4,6- dehydratase, *Escherichia coli* NM522 harbouring the gene *wcaG* for GDP-4-keto-6-deoxymannose epimerase/reductase responsible for GDP-fucose formation and *Escherichia coli* NM522 harbouring the gene *fucT* for α1,3 fucosyltransferase of *Helicobacter pylori* were transformed after 12 hours fermentation in order to produce higher amounts of GDP-4-keto-6-deoxyMan.

A 22 hours reaction beginning with mannose, GMP and fructose resulted in a GDP-L-fucose concentration of 18,4 g / L [90].

For Lewis X synthesis substrate N-acetyl lactosamine (LacNAc) was added in order to synthesize Lewis X oligosaccharides.

With this two step in vivo system based on bacterial coupling, a Lewis X concentration of 21 g / L was achieved [90].

With the invention by Koizumi et al. [90] formation of human milk oligosaccharides (HMO) such as Lewis X oligosaccharide lacto-N-fucopentaose III and other oligosaccharides like 2′-fucosyllactose, 3-fucosyllactose, Lewis a, lacto-N-tetraose, lacto-N-neotetraose, lactodifucotetraose, 3′-sialyl-3-fucosyllactose, lacto-N-fucopentaose I, lacto-N-fucopentaose

II, lacto-N-fucopentaose V, lacto-N-difucohexaose I, lacto-N-difucohexaose II, lacto-N-hexaose or lacto-N-neohexaose should be feasible.
For lacto-N-fucopentaose III production as an example for possible Lewis X synthesis by bacterial coupling, a microorganism convenient to express a *Helicobacter pylori*-derived α1,3-fucosyltransferase gene, a second microorganism which is able to synthesize GTP from GMP as well as a microorganism able to produce GDP-L-fucose from GTP and mannose is relevant.
Active α1,3-fucosyltransferases can then transfer fucose from GDP-L-fucose to the lactosamine containing substrate N-neotetraose which results in lacto-N-fucopentaose III [90].

In vivo synthesis of fucosylated human milk oligosaccharides (HMO) Lacto-N-neofucopentaose II and V (LNnFP- II and LNnFP-V) and Lacto-N-neodifucohexaose II (LND-II) in Escherichia coli by two α1,3-fucosyltransferases of Helicobacter pylori

In a study by Dumon et al. [16] it was demonstrated that successful in vivo syntheses of more than just one type of human milk oligosaccharide (HMO) are feasible.
Only human milk oligosaccharides were observed in the oligosaccharide mixtures obtained from *Escherichia coli* which might avoid expensive purifications. It was possible to express different amounts of HMO Lacto-N-neofucopentaose II and V (LNnT- II and LNnT-V) as well as Lacto-N-neodifucohexaose II (LND-II) in *Escherichia coli* JM107 derived col1FΔlacZ strains either by α1,3-fucosyltransferase FutA or FutB of *Helicobacter pylori* strain 26695.
Because of the facts that only human oligosaccharides were synthesized and different concentration patterns of certain HMO could be observed within the cells these in vivo systems might be convenient to yield combinations of distinct extracts in order to reach specific formulations.
An *Escherichia coli* JM 107 derived col1K strain was used for expression of *futA*.

Strain col1K was constructed because it shows an increased metabolic flux to GDP-L-fucose production since the *wcaJ* gene was knocked out and substituted through a kanamycin resistance gene in order to prevent synthesis of colanic acid. Furthermore this strain contains a non functional *lacZ* gene and harbours a mutated episome called *proAB* instead. Only strains with the episome *proAB* could grow on nalixidic acid rich medium and could be selected therefore [16]

The *lacZ* gene was inactivated because of α-complementation with the DE3 cassette.

Escherichia coli strain col1K F`Δ*lacZ* lacking total *β*-galactosidase was used for expression of *futB*.

Both strains, col1F`ΔlacZ and col1K could overexpress genes *IgtA* and *IgtB* from Neisseria meningitides encoding glycosyltransferases 1,3GlcNacT and 1,4GalT to produce high amounts of Lacto-N-neotetraose (LNnH) and Lacto-N-neotetraose (LNnT), the intermediate in the production of Lacto-N-neofucopentaose V (LNnFP-V) and Lacto-N-neofucopentaose II (LNnFP-II) as well as Lacto-N-neodifucohexaose II(LND-II).

LNnFP-II and LND-II show both Lewis X epitopes.

Additional lactose internalization was enabled via overexpression of lactose permease gene [16].

Fut A showed a high activity on the LNnT acceptor which resulted in a high formation of Lacto-N-neofucopentaose V (LNnFPV) first. Lacto-N-neofucopentaose V (LNnFPV) is fucosylated on the glucose residue and therefore does not include the Lewis X structure. Thus Fut A might have a preference for the reductive termini on a polylactosamine chain without Lewis X structure [16].

Since the gene *futA* contained an open reading frame responsible for a weaker expression because of a too long polynucleotide tract (poly(C)tract) a Cytosine residue was deleted [16].

Fut B in contrast shows a better activity for Lewis X structures since Lacto-N-neofucopentaose II (LNnFPII) which is fucosylated on the N-acetylglucosamine residue could be efficiently synthesized from LNnT [16].

In order to produce higher amounts of Lacto-N-neodifucohexaose II (LND-II) which is fucosylated on the N-acetylglucosamine residue of LNnT and thus contains a Lewis X structure, Fut A was metabolically engineered.

Different plasmids were tested to enable an efficient activity of FutA and FutB which were encoded by *futAon* and *futB*, respectively.

Activities are shown in table 10 [16]:

Table 10 Activity of Fucosyltransferases of *Helicobacter pylori* adapted from Dumon et al. [16]

vector	pSM9710	pFUTA1	pFUTA2	pFUTB1	pFUTB2
promotor	T7	T7	Tac	T7	Tac
gene	futA-off	futA-on	futA-on	futB	futB
% activity	10	64	100	26	4

Genes *futAon* and *futB* were separately cloned into plasmids pEXT20 or pET21 before they were separately expressed in *Escherichia coli*.

The highest activity of FutA encoded by the gene *futAon* could be reached when the gene was expressed in the Tac promoter habouring plasmid pEXT20 (pFUTA2) whereas the best activity of FutB was observed with the plasmid pET21a (pFUTB1).in which the *futB* gene was under the control of a T7 promoter [16].

A so called switch on mechanism was induced when the gene called *futAon* was transferred via pFUTA2 or pFUTA1 into *Escherichia coli* strain col1FΔlacZ which resulted in a higher expression of *futA* and production of Lewis X based oligosaccharide LNnDFH-II compared to *Escherichia coli* strains that harboured the less active gene *futA* [16].

Expression of *futA* in pFUTA2 resulted in the highest fucosylaton rate of 0,7 ± 0,2 g / L whereas expression of *futB* in pFUTB1 showed no fucosylation rate first since pFUTB1 was unstable in long time fermentations [16].

A fucosylation rate of 0,4 ± 0,2 g / L was determined when plasmid pLysS with the T7 lysosyme gene, a natural inhibitor of T7 RNA polymerase was transfected [16].

Figure 20 shows the quantitative production of HMO obtained from fermentations using either Fut A encoded by *futAon* or FutB encoded by *futB*:

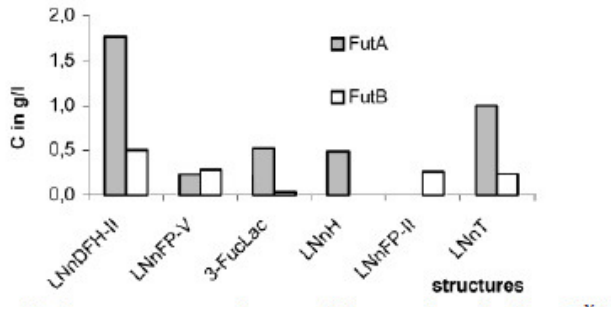


Figure 20 Concentrations of human milk oligosaccharides in fermentations with *futAon* expression (grey bars) or *futB* expression (white bars) adapted from Dumon et al. [16]

A high concentration of the Lewis X based oligosaccharide Lacto-N-neodifucohexaose II LNnDFH-II(LND-II) of 1,7 g/L fermentation broth was obtained through activity of FutA encoded by the gene *futAon* in plasmid pFUTA2 which indicates a higher fucosylating activity on the N-acetylglucosamine residue of LNnT compared to activity by FutA encoded by the less active gene *futA* [16].

In contrast to experiments with FutA encoded by the less active gene *futA*, the Lewis X based oligosaccharide Lacto-N-neodifucohexaose II represented the main product and not Lacto-N-neofucopentaose LNnFP-V which occurred only in small concentrations [16].

According to Dumon et al. [16] it is therefore conceivable that Lacto-N-neofucopentaose (LNnFP-V) served as an intermediate for the synthesis of Lacto-N-neodifucohexaose II (LNnDFH-II).

Interestingly a significant amount of 3`-fucosyllactose could be also produced which could stand for an additional FutA activity on lactose beside LNnT.

However the study by Dumon et al. [16] demonstrates that pure production of sole human milk oligosaccharides in different amounts is principally possible and might be an efficient method to yield extracts that can be added to infant formulas for instance.

In vivo synthesis of fucosylated human milk oligosaccharides (HMO) Lacto-N-neofucopentaose I and 2`-fucosyllactose in Escherichia coli by Helicobacter pylori fucosyltransferase FutC and Neisseria meningitis transferases

In a large scale production by Droullard et al. [15] the human milk oligosaccharides (HMO) Lacto-N-neofucopentaose I (LNnF-1), a H-2 antigen oligosaccharide and 2`-fucosyllactose, the glucose analog of H-2 antigen could be synthesized via overexpression of the *Neisseria meningitis* genes *igtA* and *igtB* as well as the *Helicobacter pylori* gene *futC*.
Genes *igtA* and *igtB* code for β1,3-N-acetylgucosaminyltransferase and β1,4-galactosyltransferase, respectively, and *futC* for α1,2-fucosyltransferase FutC.
Since the *futC* gene contained an open reading frame with a 14 C tract which led to an inactive truncated variant the 14 C tract was replaced by a 12 nucleotide sequence with nine cytosine and three additional adenosine nucleotides in order to eliminate three consecutive rare CCC codons and to avoid mutations that are observed in homopolymeric tracts [15].
For Lacto-N-neofucopentaose I (LNnF-1) the *Escherichia coli* strain Col1F`ΔlacZ,DE3 was transfected with plasmids pET-21afutC harbouring the *futC* gene and pLNTR, which contained the *Neisseria meningitis* genes *igtA* and *igtB* as well as *rcsA* gene.
The strain was then called SD5 [15].
One opportunity to avoid consumption of GDP-L-fucose to colonic acid is to overexpress the *rcsA* gene of the colonic acid operon which codes for the positive regulatory protein and transcriptional activator RcsA [81-83].
2`-fucosyllactose and lacto-N-fucopentaose I could be synthesized from LNnT whereby mainly the H type 2 antigen oligosaccharide lacto-N-fucopentaose I was produced [15].
To obtain higher concentrations of lacto-N-fucopentaose I one opportunity could be to delay *futC* expression until lacto-N-neotetraose is already synthesized.
Conversely it should be possible to produce only 2`-fucosyllactose by a strain called SD6 which only expresses *futC* and *rcsA*.
Concentration data for 2`fucosyllactose and Lacto-N-neofucopentaose I of the fed batch fermentation were not available [15].

In vivo synthesis of α1,2-fucosyllactose and fucosylated-complex human milk oligosaccharides (HMO) by fucosyltransferases from other origins than Helicobacter pylori

Although FucT2 from *Helicobacter pylori* is probably the most frequent used α1,2-fucosyltransferase in the production of 2`-fucosyllactose [13,81] fucosyltransferases from other bacterial origins, animals or even humans were determined and tested on substrate specificity in vivo.
The following examples demonstrate the applicability of fucosyltransferases from other origins than *Helicobacter pylori* in the production of α1,2-fucosyllactose and fucosylated-complex human milk oligosaccharides (HMO).

Use of human α1,2-fucosyltransferase FucT I (Fut1) in the in vivo synthesis of α1,2-fucosyllactose in transgenic animals

Human α1,2-fucosyltransferase Fut1 or synonymous FucT I whose substrate specificity and occurence was precisely described in chapter 1 could be expressed in transgenic animals such as rabbit, goat, pig or mouse to yield 2`-fucosyllactose.
Through an invention by Prieto et al. [42] the production of homologous or heterologous structures can be achieved.
To produce these structures three steps are important:
First the desired transgene must be prepared and isolated from a particular eukaryotic or prokaryotic organism.
Second the transgene which was inserted into a vector with a promoter and mRNA processing signal (for example a polyadenylation signal) is transferred respectively the transgenes that were separately inserted into vectors with promoter and mRNA processing signal (for example polyadenylation signal) are transferred into the pronucleus of a non human but mammalian embryo of the same or of a different mammalian species.

And third the milking of the non human mammal is essential to get the milk which is rich on oligosaccharides and glycoproteins [42].
Step three includes the identification of at least one female offspring which might give evidence that there was no negative interference between the transgene(s) and mammary gland development.
A functional expression of the transgene(s) enables the production of enzyme(s) which (that) is (are) able to perform the synthesis of glycoproteins or oligosaccharides such as fucosylated HMO.
The transgene contains at least one expression regulatory sequence and a signal sequence that must both show functionality in mammary secretory cells as well as a sequence encoding the enzyme responsible for the production of desired glycoprotein or oligosaccharide.
Each transgene is linked to a mammary gland-specific promoter to enable the expression of the oligosaccharide(s) or glycoprotein(s) in the milk of the mammal [42].
Vectors that can be used are plasmids, cosmids, phage DNA, viruses or vectors specific for yeasts, plants, mammalians and other hosts [42].
Promotors that can efficiently introduced are lactogenic promoters like lactalbumin, casein or whey acidic protein promoters and can be linked to different processing signal sequences that are polyadenylation signals such as mammalian or viral signals of SV-40T-antigen, avalbumin or bovine growth hormone (bGHpdyA) [42].
Different animals such as mouse, rat, rabbit, pig, goat, sheep or cow come into question to represent a transgenic animal for transgenic syntheses [42].
Enzymes that can be introduced can be glycosyltransferases like fucosyltransferases such as FucT I, FucT II, FucT III or FucT IV [42]. Thus the production of fucosylated oligosaccharides is possible by this invention.
Fucosylated oligosaccharides that can be synthesized are 2`-FL, 3`-FL, LNF-I, LNF-II, LNF-III, LNF-IV, LNF-V or sialylated derivatives of LNF-I, LNF-II, LNF-III, LNF-IV and LNF-V. Furthermore fucosyloligosaccharides lacto-N-difucopentaose I and II, sialylated derivatives of lacto-N-difucopentaose I and II as well as fucosylated derivatives of lacto-N-hexaose and fucosylated derivatives of sialyltetrasaccharides a, b and c can be produced [42].
One or more fucosyltransferases can be expressed in the mammary gland and thus one or more desired fucosylated oligosaccharides can be synthesized and enriched in a milk fraction of the transgenic animal [42].
Milk fractions with the desired human milk oligosaccharide(s) are obtained through evaporation, lyophilisation, crystallisation, ultrafiltration, dialysis or different types of

chromatography such as affinity, anion exchange or gel exclusion electrophoresis and other methods [42].

In the patent by Prieto et al. [35] it could be demonstrated that expression of the gene for the exogenous human glycosyltransferase FucT I or synonymous H-α-1-2 fucosyltransferase under the control of the lactogenic murine whey acidic protein promoter could shield its expression from tissues other than the mammary gland and prevents lethal modifications of the glycoconjugates as a result.

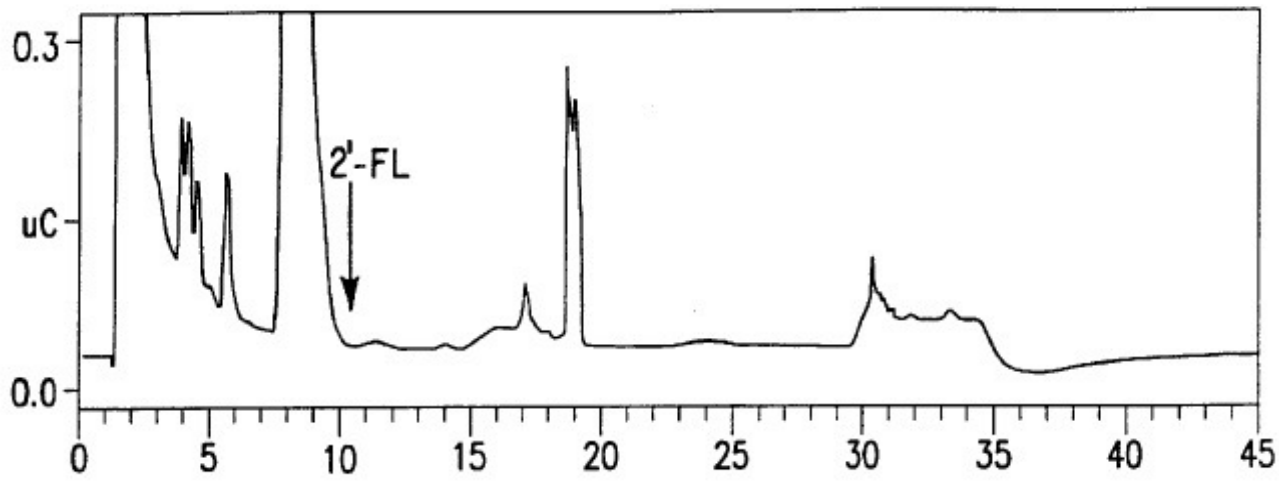
Zygotes were microinjected with a linear DNA fragment which contained a transcriptional regulatory region with the murine whey acidic protein promoter as well as a cDNA, coding for the fucosyltransferase FucT I gene and a gene including the polyadenylation signal sequence of bovine growth hormone.

The zygotes were transferred into oviducts of synchronized, pseudopregnant 5 till 6 month old recipients one hour after microinjection [35].

Milk samples from the pubs of control rabbits and transgenic rabbits were collected, analyzed and compared with focus on 2`-fucosyllactose production via oligosaccharide analysis by High-Pressure Anion-Exchange Chromatography (HPAEC) [35].

Furthermore a so called positive spiked sample was prepared and analyzed which contained 2`-fucosyllactose from transgenic rabbit and 2 µL 1000 ppm 2`-fucosyllactose since it was not clear if 2`-fucosyllactose concentration of the pure sample of the transgenic rabbit was under the detection limit.

Chromatographic profiles of non transgenic control rabbit, transgenic rabbit and spiked positive transgenic rabbit milk samples are depicted in figure 21 adapted from Prieto [35]:



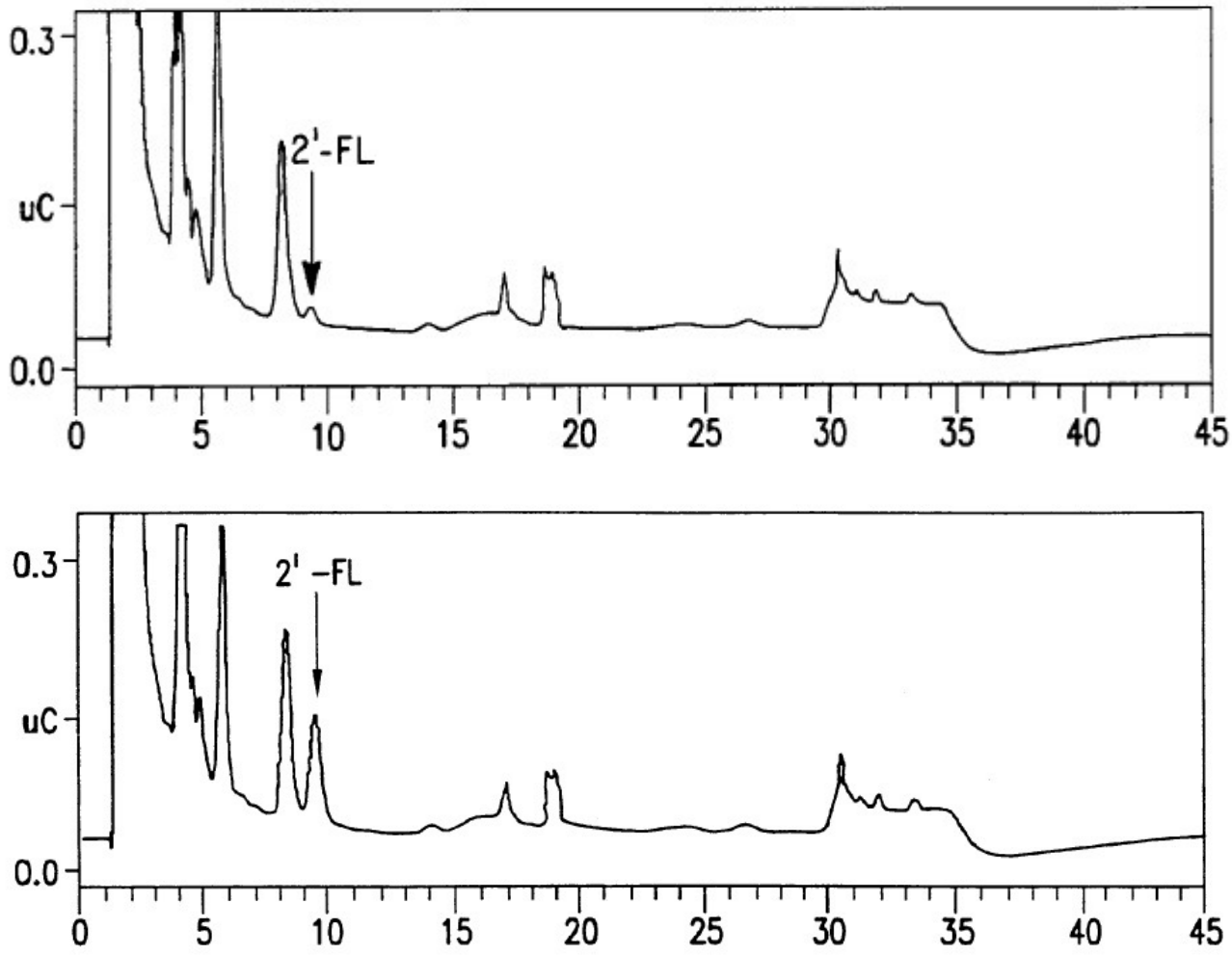


Figure 21 Oligosaccharide profiles of milk samples obtained from control and transgenic rabbit as well as oligosaccharide profile of spiked milk sample adapted from Prieto [35]

The first panel shows the oligosaccharide profile observed in the milk sample of the control rabbit, the second one the oligosaccharide profile of the transgenic one and the third the oligosaccharide profile of the spiked milk sample of the transgenic rabbit.
The pure milk sample of the transgenic rabbit contained detectable 2`-FL amounts which was corroborated by the spiked profile [35].
The oligosaccharide profiles reveal that a significantly higher lactose concentration of lactose was observed in the control sample compared to the spiked milk sample of the transgenic rabbit and the pure milk sample of the transgenic rabbit which indicates that in these samples lactose was efficiently converted to 2`-fucosyllactose and probably other products with fucose-α1,2-linkages such as fucosylated glycoproteins or other fucosylated oligosaccharides.

In the review by Prieto [35] it was reported that after the day of parturation no further 2`-fucosyllactose amounts were detectable in the chromatographic profiles of a transgenic rabbit as can be seen in figure 22 adapted from Prieto [35]:

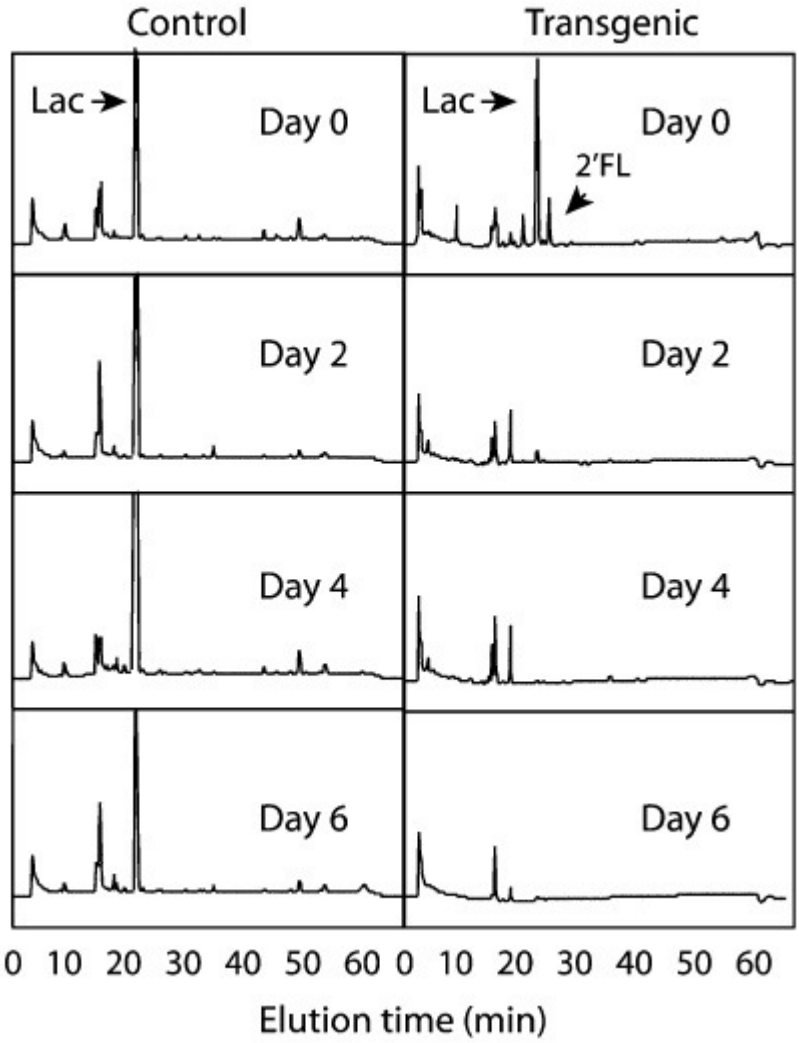


Figure 22 Chromatographic oligosaccharide profiles of transgenic rabbit (right) and non-transgenic control rabbit (left) from four measurements. Lac, Lactose signal. 2`FL. 2`-fucosyllactose signal adapted from Prieto [35]

8 chromatograms of oligosaccharide extracts from rabbit milk samples are depicted. On the left of figure 22 a series of chromatograms obtained from samples of a non-transgenic rabbit can be seen which does not produce 2`-fucosyllactose.
On the right equivalent chromatograms of a transgenic rabbit which expresses FucT1 and is able to synthesize 2`-fucosyllactose are depicted.
The upper panel in this series refers to milk from the day of parturition (day 0) whereas the lower panel corresponds to 8 days after parturation.

As can be seen in the top chromatogram, signals corresponding to 2`-FL as well as lactose are clearly visible in the transgenic rabbit on the day of parturition (day 0).

But only trace amounts of lactose were found 48 hours after perturation and 2`-fucosyllactose was even not detectable after 24 hours and 48 hours as can be seen in the second panel from above which leads to the assumption that there is no α1,2-fucosyltransferase activity after 24 hours for the production of 2`-FL [35].

Interestingly a significant increase in the production of fucosylated proteins with α1,2 linkages, except on day 2 , was observed via Western Blot analysis in the transgenic rabbit which refers to a continuous activity of α1,2-fucosyltransferase FucT I whereas milk proteins from the non transgenic control rabbit showed no detectable Fucα1-2 linkages.

According to Prieto et al. [35], the transgene for FucT I could have been interfered with the lactation process which could be an explanation for the strong decrease in lactose concentration and no detectable 2`-FL after one day of perturation.

A protein similar to the protein clusterin which is known to be involved in protection from apoptosis and cell differentiation was identified. This observation reveals that proteins such as clusterin could have been directly overglycosylated or overexpressed because of FucT I activity in order to arrest the lactation process and to prohibit 2`-fucosyllactose production [35].

However, it could be demonstrated that a production of 2`-fucosyllactose in transgenic rabbits is principally feasible on the day of perturation but further investigations such as metabolic engineering might be important to improve synthesis.

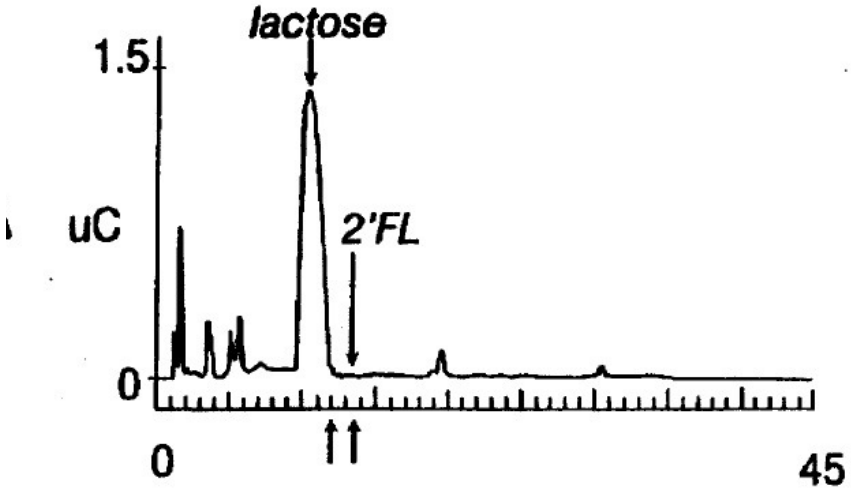
Expression of *fucTI* for α1,2-fucosyltransferase was also possible in the mammary gland of transgenic mice [12].

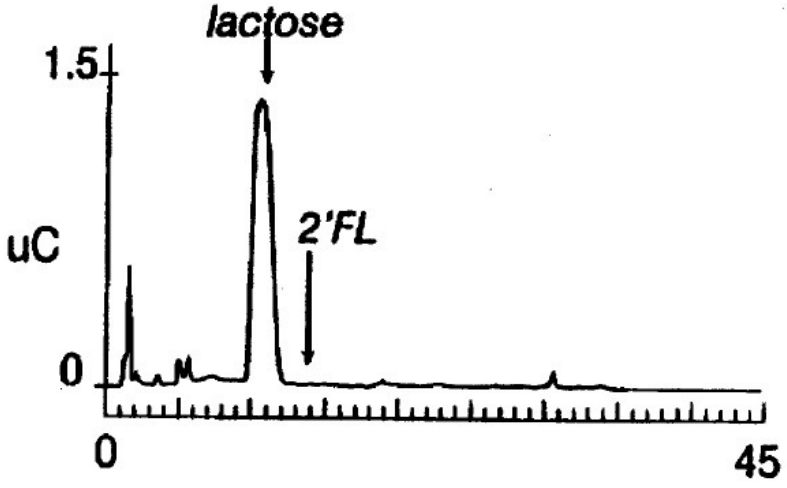
A cDNA encoding α1,2-fucosyltransferase was isolated from the epidermal carcinoma cell line A431 cDNA library.

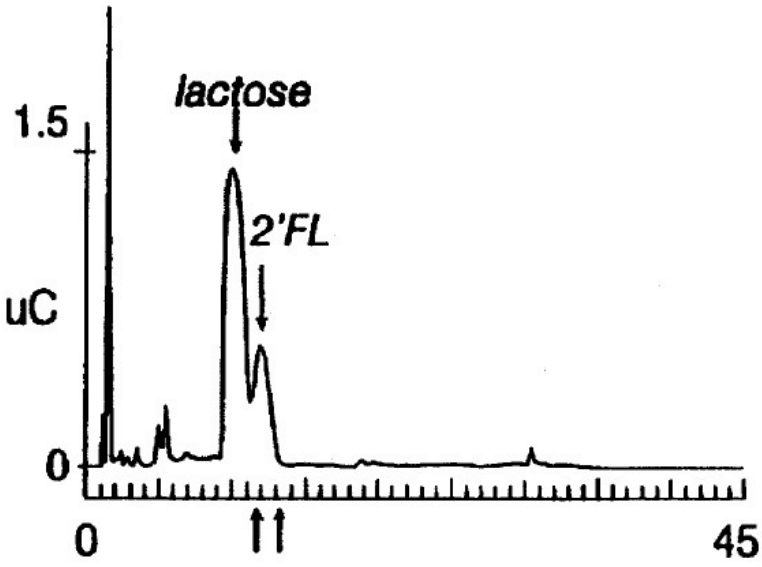
An α1,2-fucosyltransferase cDNA genetic construct under the control of the murine whey acidic promoter (WAP) of a mouse and with a polyadenylation signal sequence was microinjected at concentrations into male pronuclei of mice embryos in order to implant them into pseudo pregnant mice which had recently mated with sterile males [12].

Milk samples from the pubs of control mice and transgenic mice were collected, analyzed and compared with focus on 2`-fucosyllactose production via oligosaccharide analysis by High-Pressure Anion-Exchange Chromatography (HPAEC) [12].

In figure 23 the chromatographic profiles of milk samples by two control mice and four transgenic mice are depicted [12]:


lactose
1.5
uC
2'FL
0
0
45


lactose
1.5
uC
2'FL
0
0
45


lactose
1.5
uC
2'FL
0
0
45

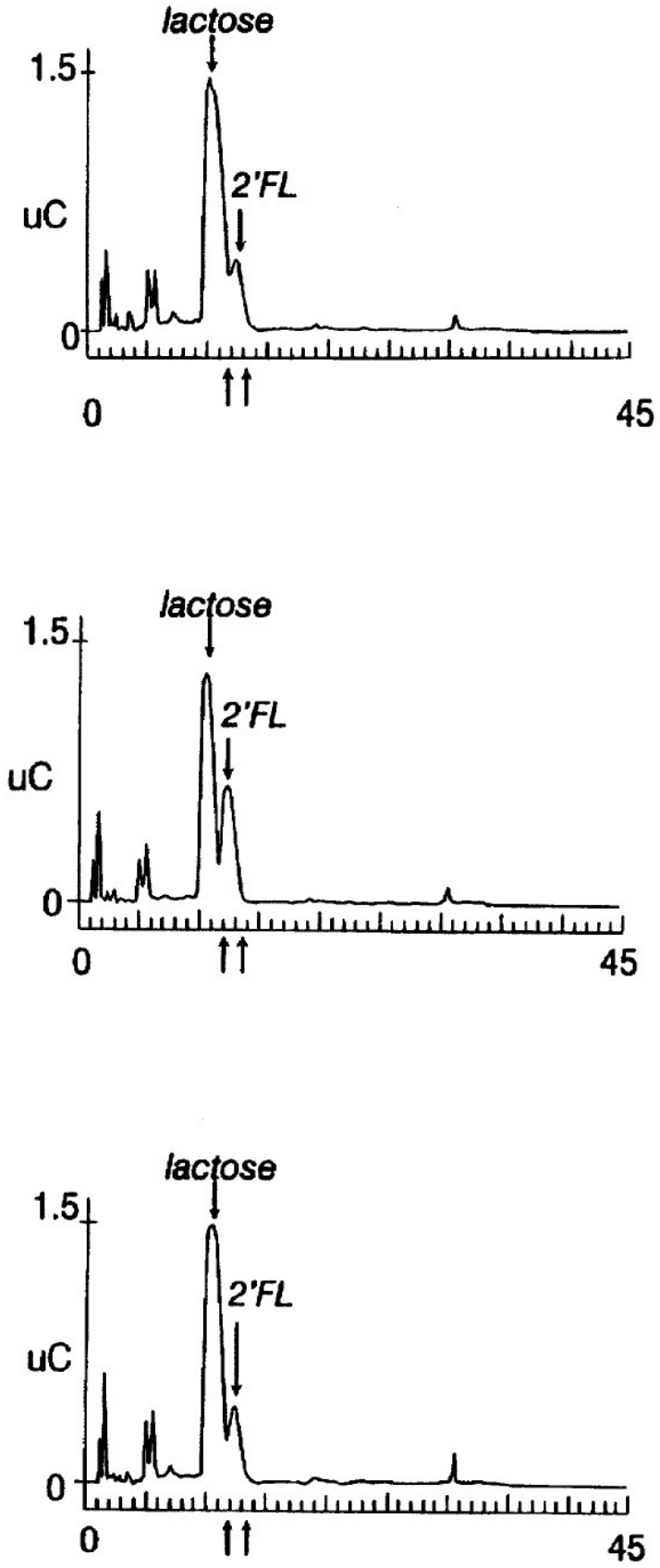


Figure 23 Chromatographic profiles from control mice and transgenic mice adapted from Prieto et al. [12]

The upper two chromatographic profiles belong to two control mice whereas the following four are those observed from transgenic mice.
In all four transgenic mice 2`-fucosyllactose formation could be observed since they showed an α1,2-fucosyltransferase activity.
By means of the chromatographic peak areas concentration of 2`-fucosyllactose in the transgenic mice was calculated [12]. Values are depicted in table 11:

Table 11 Concentrations of 2`-fucosyllactose non-transgenic control and transgenic mice adapted from Prieto et al. [12]

Donor	2`-fucosyl-lactose concentration (mg/L)[b]
1. Control (nontransgenic)	0
2. Transgenic	
28-29	711
29-119	468
34-34	686
72-66	338

The four transgenic animals are referred to in Fig.5 as 28-89, 29-119, 34-34 and 72-66, respectively.
The highest concentration of 711 mg / L milk sample was referred to transgenic animal 28-29 [12] which is irritating because milk samples obtained from the transgenic mice and control mice were in a range between 90 and 100 µL. Therefore an amount just 71 µg 2`-fucosyllactose in the sample of transgenic animal 28-29 could be actually obtained.
The question arises if mice are the convenient organisms for HMO production in high quantities. What is more, mouse milk is known to be very expensive.
Organisms such as cows might be better convenient for further metabolic engineering in order to reach an efficient production of 2`-fucosyllactose or other HMO since a single cow can produce more than 10.000 litres of milk [92].
However, it is visible that the peak areas of 2`-fucosyllactose observed in the chromatographic profiles of transgenic mice were significantly higher than those of the transgenic rabbit which leads to the assumption that product concentration in transgenic mice was significantly higher.

It could be an indication that there was probably no negative interfering between the α1,2-fucosyltransferase gene construct and the lactation process within the transgenic mice.

Summary of microbial in vivo syntheses of various human milk oligosaccharides (HMO)

In table 12 concentrations of fucosylated human milk oligosaccharides as well as GDP-fucose that were achieved by different in vivo systems and discussed in this chapter are summarized:

Table 12 In vivo production of human milk oligosaccharides (HMO)

Bacterial-strains / mammals	*Fucosyltransferases*	*Fucosyltransferase source*	*Substrate*	*Cultivation*	*Desired prouct(s)*	*Maximal concentration of desired product(s) (g / L)*	*Reference number*
E. coli JM 109	α 1,2-fucosyltransferase FutC	*Helicobacter pylori*	Lactose	Fed batch	2`-FL	20,28 ± 0,83	[13]
E. coli JM109DE3	α 1,2-fucosyltransferase FutC	*Helicobacter pylori*	Lactose	batch	2`-FL	1,23 ± 0,011	[81]
E. coli NM522 *C.ammoniagenes*	α 1,3-fucosyltransferase	*Helicobacter pylori*	LacNAc	batch	Lewis X	21	[89]
Col1FΔlacZ	α 1,3-fucosyltransferase FutAon	*Helicobacter pylori*	LNnT	batch	LNnDFHII	1,7	[16]
Col1FΔlacZ	α 1,3-fucosyltransferase FutB	*Helicobacter pylori*	LNnT	batch	LNnFPII LNnFPV	0,25 0,25	[16]
Col1FΔlacZDE3	FutC	*Helicobacter pylori*	LNnT lactose	Fed batch	LNnFPI 2´-FL	No data available	[15]
Transgenic rabbit	FucTI	Human	lactose	-	2`-FL	No data available	[35]
Transgenic mice	FucTi	Human	lactose	-	2`-FL	0,711	[12]

Various in vivo systems were discussed in this chapter in order to show the broad range of application of whole cell catalysts respectively transgenic animals and its importance in the large scale synthesis of fucosylated human milk oligosaccharides (HMO).
Further investigations may be useful and essential to improve product yields and introduce new, more efficient metabolic pathways to obtain high amounts of certain human milk oligosaccharides (HMO).

Enzymatic in vitro synthesis of fucosylated human milk oligosaccharides (HMO), core structures thereof as well as GDP-L-fucose

The in vitro synthesis of carbohydrate structures is often complicated by the cost and difficulty in obtaining reactants.
Different types of enzymes, namely so called retaining α-L-fucosidases, α-L-fucosynthases and α-L-fucosyltransferases were identified to be able to synthesize fucosylated oligosaccharides in vitro but each of them showed certain disadvantages concerning abundance, specificity or product yield [88].
Retaining α-L-fucosidases belong to the glycosylhydrolases and show transfucosylating but mainly hydrolising activity.
Hydrolising activity is based on a double displacement process within the active centre in which a nucleophilic residue first attacks the anomeric center and a glycosyl-enzyme intermediate is achieved when a leaving group is protonated through an acid [93].
The deprotonated acid functions as a base and thereby activates an incoming water molecule. This process leads to the hydrolysis of the glycosyl-enzyme intermediate and to a formation of a product with the same anomeric configuration as the substrate [93].
If suitable acceptors enter the catalytic site instead of a water molecule, then transglycosylation is possible and the desired fucosylated product can be synthesized.
In this case the glycone of the glycosyl enzyme is transferred to an acceptor sugar rather than to water [92] but specificity and yield- which are normally not convenient for larger scale productions- are generally lower compared to the synthesis by fucosyltransferases [93].
The main problem concerning the use of these glycosidases is mostly based on the fact that the desired product of the reaction is coincident a substrate for the enzyme because of its hydrolysing activity and therefore product yields are generally rather low [93].
Reaction conditions [94] and often directed evolution as was important for α-fucosidases from Thermotoga maritima [95] need to be optimized to reduce hydrolysis.
Sometimes also natural α-fucosidases without further genetic modifications such as AlfC from *Lactobacillus casei* show a strong activity concerning fucosylation as well as low

hydrolysing activity as could be demonstrated by the high product yield of 56% of fucosyl-α-1,6-N-acetylglucosamine, the maximum product yield described for a wild-type fucosidase. The efficient formation of fucosyl-α-1,6-N-acetylglucosamine led to the conclusion that this disaccharide may not be the natural substrate of AlfC [96].

One advantage of glycosidases compared to glycosyltransferases is the high solubility and broader specificity of donor and acceptor substrates [97].

Furthermore they can use more stable and commercially available donor substrates compared to glycosynthases which need unstable *α*-glycosyl fluoride as the donor substrate.

Hydrolysis of *α* -glycosyl fluoride seems to be responsible for low product yields by glycosynthases since it competes with the transglycosylation reaction, especially at high temperatures [97].

Through the conversion of a fucosidase into a fucosynthase the hydrolysing function of the natural occurring enzyme fucosidase is avoided. Beside the unstable donor *α*-glycosyl fluoride required for successful synthesis a further drawback of the fucosynthase technology is the missing commercial availability of fucosynthases in large quantities [93].

Although inverting glycosylhydrolases respectively fucosidases are not able to achieve transglycosylating products they can be converted to glycosynthases that are able to catalyze the formation of these products.

Inverting glycosylhydrolases show a single displacement mechanism based on a water molecule activated by a certain base that attacks the anomeric centre and a concomitant protonation of a glycosyl oxygen by a general acid. Thereby one carboxylic acid residue acts as general base and the other as general acid catalyst.

As a result a bond cleavage and formation of a product with an inverted anomeric configuration are obtained [93].

Two strategies have been suggested for the construction of an inverting glycosynthase from an inverting glycosidase in order to inhibit hydrolysis but to reach formation of products such as 2`-fucosyllactose by transglycosylation.

The first one is based on a replacement of a general base residue with a non-catalytic residue, the second on a replacement of a residue holding a non-polar residue with a catalytic water molecule as was shown in a study by Wada et al. [93] where a maximum product yield of 2`-fucosyllactose could be obtained by the replacement of asparagine aspartic acid-766 to glycine in the inverting 1,2-α-L-Fucosidase AfcA of *Bifidobacterium bifidum*.

Since aspartic acid-766 (D766) was replaced through glycine it was not able to activate asparagine-423 (N423) acting as a base and hydrolysis of 2`-fucosyllactose was avoided.

As a result inverting glycosynthases catalyze the synthesis of glycoconjugates when a glycosyl fluoride donor of opposite configuration to the natural substrate is available.
The glycosyl-enzyme intermediate is mimicked by the donor glycosyl fluoride and its sugar moiety transferred to the acceptor sugar.
Catalytic reactions by glycosidases and glycosynthases are depicted in figure 24 adapted from Hancock et al. [98]:

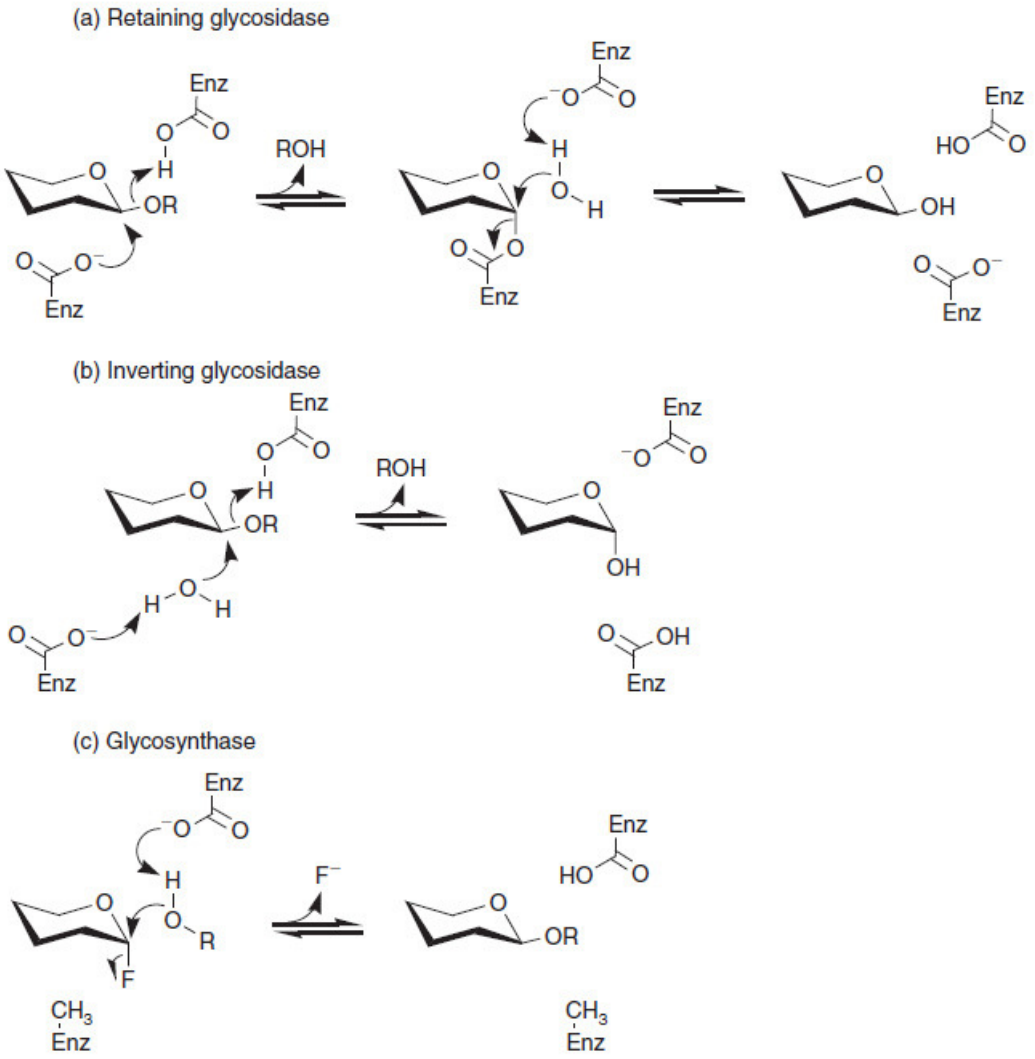


Figure 24 Catalytic mechanism by retaining and inverting glycosidase as well as glycosynthase adapted from Hancock et al. [98]

A third enzymatic process for the synthesis of HMO is based on α-1,2-fucosyltransferases but these enzymes are dependent on availabilities of expensive sugar nucleotide type donors.

Therefore fucosyltransferases may be rather convenient for in vivo systems where they act as whole cell catalysts because optimal conditions do exist within the cell and sugar nucleotide type donors can be efficiently regenerated [80;81].
A further disadvantage of fucosyltransferases for the in vitro approach is that they are often membrane-bound and thus difficult to isolate and purify [99].
In general it can be said that whole cell catalysts based on fucosyltransferase activity may be rather convenient to enable a less cost intensive and more efficient synthesis of human milk oligosaccharides and other compounds at large scale.
Nevertheless the production of human milk oligosaccharides by other strategies than in vivo systems will be discussed in the following examples.
Last but not least the enzymatic synthesis of GDP-L-fucose will be described since GDP-L-fucose represents an essential intermediate in the synthesis of human milk oligosaccharides (HMO).

Usage of fucosidases in the enzymatic synthesis of human milk oligosaccharides(HMO) or core structures thereof

The following examples give a summary of the in vitro use of retaining α-L-fucosidases involved in the formation of various fucosylated human milk oligosaccharides or core structures thereof.
Thereby the α-L-fucosidases were either used in their natural occurring structure or genetically engineered.

Example 1: The use of α-L-fucosidase of Penicillium multicolour in the enzymatic synthesis of a core structure found in Lewis X based oligosaccharides LNFP III and DFLNH a

Lewis X based human milk oligosaccharides (HMO) such as Lacto-N-fucopentaose (LNFP-III) and difucosylated lacto-N-hexaose a (DFLNH a) contain the vital core unit and disaccharide
α -L-Fuc-(1,3)-GlcNAc as can be seen in table 2 with structures adapted from Kobata et al. [18] and Kunz et al. [27].
In a previous report by Ajisaka et al. [100] an α-l-fucosidase from *Aspergillus niger* was identified to be able to produce α -L-Fuc-(1,3)-Glc or α -L-Fuc-(1,3)-GlcNAc in high regioselectivity and yield.
A better alternative with regard to enzyme stability in organic co-solvents was found in α-L-fucosidase of *Penicillium multicolour* in order to produce higher amounts of the mentioned core structures [101].
It could be demonstrated that α-L-fucosidase of *Penicillium multicolour* was convenient for the in vitro production of isomeric pure, fucosylated disaccharides α -L-Fuc-(1,3)-GlcNAc and α -L-Fuc-(1,3)-D-Glc.
Production of isomeric pure α -l-Fuc-(1,3)-D-GlcNAc was achieved by a reaction mix containing partially purified enzyme α-L-fucosidase (6,2 units) from *Penicillium multicolour* as well as pNP- α -Fuc and D-GlcNAc as donor respectively acceptor for the enzyme.
The enzyme was partially purified from a so called “Lactase-P” freeze dried powder of *Penicillium multicolour* which contained enzyme α-L-fucosidase from *Penicillium multicolour*.
Further on the reaction was performed in 0,1M sodium acetate buffer containing 10% DMSO at a pH of 5 and a temperature of 37°C [101].
The product yield of α -l-Fuc-(1,3)-D-GlcNAc using enzyme α-L-fucosidase from *Penicillium multicolour* was 49,3% which correlated to an isolated amount of 63,5 mg from the described reaction mix by activated carbon column chromatography [101].
It was more or less equal to those of *Aspergillus niger* which was 58% [100;101].
Production of isomeric pure α -l-Fuc-(1,3)-D-Glc was achieved by a reaction mix containing “Lactase-P” from *Penicillium multicolour* and therefore crude enzyme as well as pNP- α -Fuc and Glc as donor respectively acceptor for the enzyme.

Further on the reaction was performed in 0,1M sodium acetate buffer containing 10% DMF at a pH of 5 and a temperature of 37°C [101].

Product yield of α -l-Fuc-(1,3)-D-Glc using enzyme α-L-fucosidase from *Penicillium multicolour* was 27.9% which correlated to an isolated amount of 35,2 mg from the reaction mix by activated carbon column chromatography [101].

It was significantly lower than those of α-L-fucosidase from *Aspergillus niger* which was 61% [100].

The better stability and longer lasting activity in organic solvent of α-L-fucosidase from *Penicillium multicolour* [101] compared to α-l-fucosidase from *Aspergillus niger* [100] enables a stronger dissolving of pNP- α-L-Fuc since pNP- α-L-Fuc is worse dissolved in aqueous buffer based systems.

For instance a better stability in organic solvent of α -L-fucosidase from *Penicillium multicolour* [101] could be observed in a 20% DMSO solution where a constant activity on pNP- α-L-Fuc was measured whereas the α-L-fucosidase from *Aspergillus niger* [100] lost 33% of activity in the equal solution after 3 hours reaction.

Activities on pNP- α-L-Fuc of α -L-fucosidase from *Penicillium multicolour* and *Aspergillus niger* tested in different organic solvents are compared in table 13 [101]:

Table 13 Dependence of α-L-fucosidase activity on organic cosolvents adapted from Ajisaka et al. [101]

Organic solvent	Concentration (v/v%)	Residual activity with *A.niger* (%)	Residual activity with *P.multicolor* (%)
DMSO	10	97	100
	20	68	100
	30	20	58
DMF	10	100	100
	20	0	71
	30	0	1
Dioxane	10	96	100
	20	14	55
	30	0	0

According to table 13 it is obvious that α -L-fucosidase from *Penicillium multicolour* [101] is averaged more stable in organic solvents.

The highest activity could be determined at a pH of 5 using different buffers as shown in figure 25 (A).
In figure 25 (B) it is visible that the enzyme was stable in a pH range from 3 to 7 [101]:

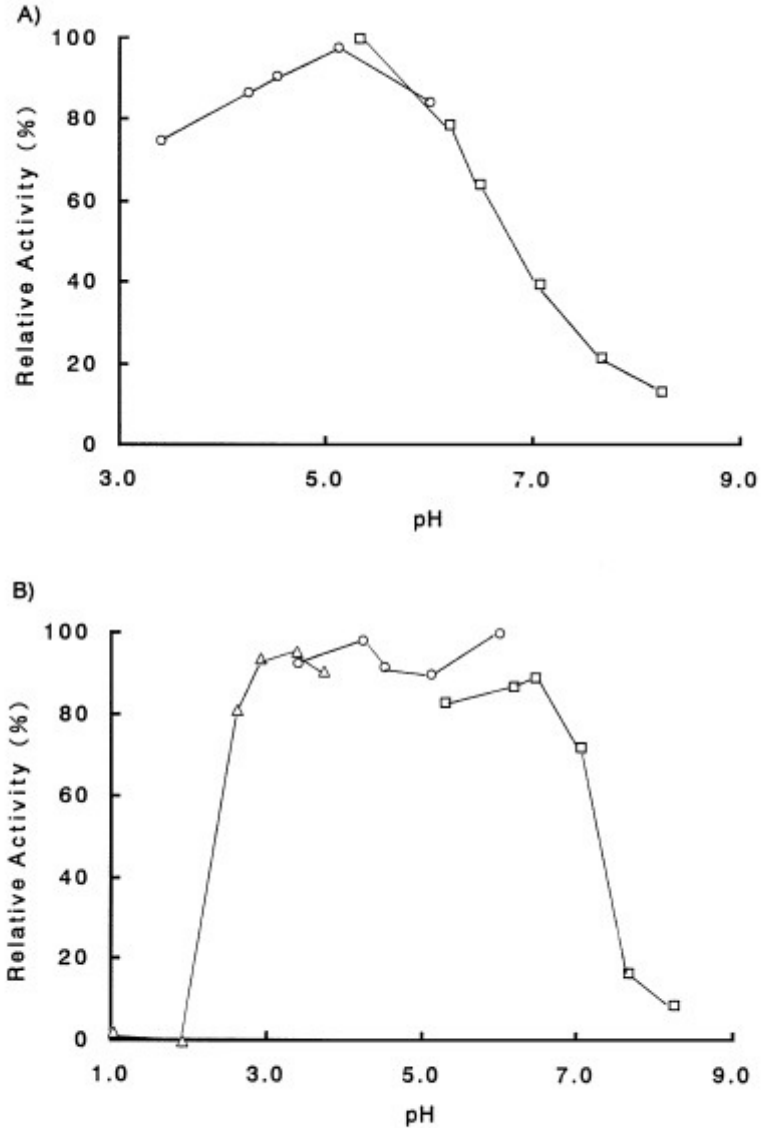


Figure 25 Optimum pH (A) and pH stability (B) adapted from Ajisaka et al. [101]. Triangles stand for activities measured in 0,1 M glycine buffer, circles for activities measured in 0,1 M acetate buffer and squares for activities measured in 0,1 M phosphate buffer

Stability with focus on temperature was ensured for temperatures up to 50°C and was almost equal to those observed for α -l-fucosidase from *Aspergillus niger* [100;101].
Therefore a successful reaction to produce fucosylated disaccharide α -L-Fuc-(1,3)-D-GlcNAc could be achieved at 37°C [101].

According to figure 26 no peak corresponding to the regio-isomer around the peak of α -l-Fuc-(1,3)-D-GlcNAc (peak C) is visible in a chromatogram received from HPLC which leads to the conclusion that only isomeric pure α -l-Fuc-(1,3)-D-GlcNAc was synthesized by α -L-fucosidase from *Penicillium multicolour* [101].

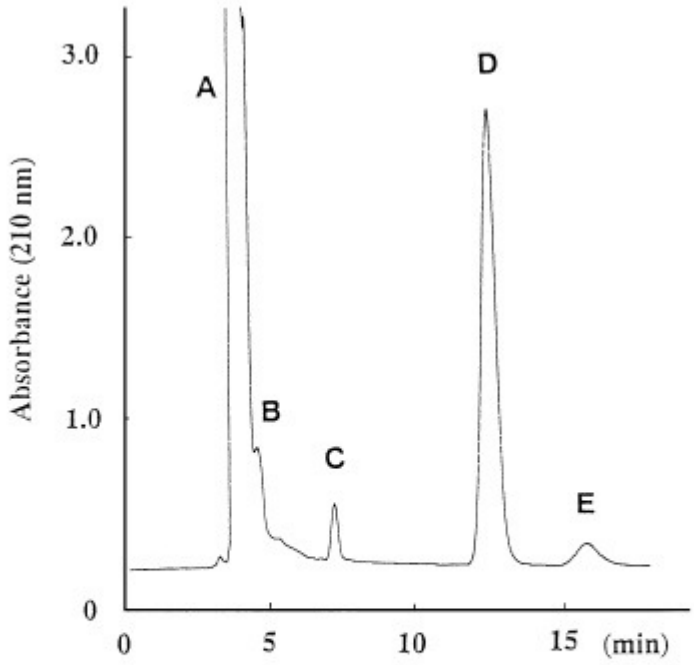


Figure 26 Chromatogram of the HPLC 3 hours after the reaction start adapted from Ajisaka et al. [101]. A, B, C, D and E represent pNP-α-Fuc, Fuc, α -L-Fuc-(1,3)-D-GlcNAc, GlcNAc and p-nitrophenol, respectively

Although this enzyme might be also able to synthesize α -L-Fuc-(1,6)-D-GlcNAc and α -L-Fuc-(1,2)-D-Gal through its fucosylating activity on D-GlcNAc respectively D-Gal , only α -L-Fuc-(1,3)-D-GlcNAc could be obtained from reaction on D-GlcNAc since the hydrolysing activity on α -L-Fuc-(1,6)-D-GlcNAc and α -L-Fuc-(1,2)-D-Gal was stronger and similar to donor pNP- α -Fuc as shown in figure 27 adapted from Ajisaka et al. [101]:

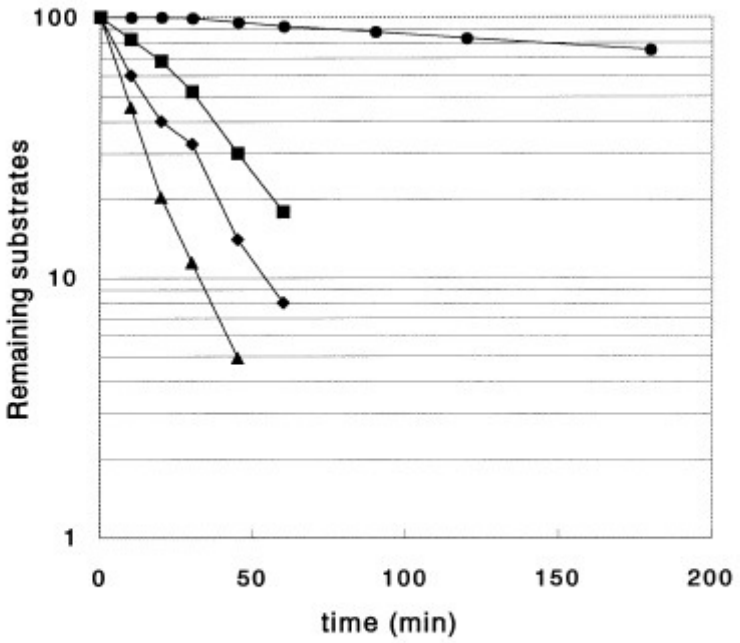


Figure 27 Relative rate of hydrolysis of pNP-α-Fuc (symbolized as triangle), α -L-Fuc-(1,2)-D-Gal (symbolized as rhombus), α -L-Fuc-(1,3)-D-GlcNAc (symbolized as circle) and α -L-Fuc-(1,6)-D-GlcNAc (symbolized as square) adapted from Ajisaka et al. [101]

As a result α -L-Fuc-(1,6)-D-GlcNAc or α -L-Fuc-(1,2)-D-Gal might be hydrolysed as soon as they were formed through the fucosylating activity of this enzyme and thus α -L-Fuc-(1,3)-D-GlcNAc can be obtained as pure product after reaction time [101].

There exist also two different α-L-fucosidases from *Aspergillus niger* [100] that both catalyze α-L-1,3 linkages and show further hydrolysing activity, one which is responsible for a selective cleaving of α-L-1,2 linkages and the other which is responsible for selective cleaving of α-L-1,6 linkages.

Ajisaka et al. [100] were able to demonstrate that the synthesis of 3-O- α-L-fucopyranosyl-D-glucose was possible with those α-L-fucosidase from *Aspergillus niger* which cleaves α-L-1,2 linkages but fails to cleave α-L-1,6 linkages.

A 12 hour incubation at 37°C of nitrophenyl α -L-fucopyranoside and D- glucose dissolved in 0.1M acetate buffer at phH 5.0 and N,N-dimethylformamide as well as semipurified α-L-fucosidase from *Aspergillus niger* resulted in the formation of 3-O- α-L-fucopyranosyl-D-glucose [100].

A yield of 61 % was obtained which correlated to a purified amount of 17,5 mg by activated carbon column chromatography [100].

When α-L-fucosidase from *Aspergillus niger* and nitrophenyl α -L-fucopyranoside were incubated with acceptor 2-acetamido-2-deoxy-D-glucose in the reaction mix instead of D-glucose for 12 hours and at 37°C, the product was 2-acetamido-2deoxy-3-O-α –L-fucopyranosyl-D-glucose [100].
A yield of 58% was achieved which correlated to 17,3 mg purified product by activated carbon column chromatography [100].
In contrast to α-L-fucosidase from *Aspergillus niger* [100], α-L-fucosidase from *Corynebacterium* [100] was used to catalize α1,2-linkages through its fucosylating activity.
Substrate methyl β-D-galactopyranoside could be synthesized by α-L-fucosidase from *Corynebacterium* to methyl 2-O- α -L-fucopyranosyl-β-D-galactopyranoside by incubation of the following reaction mix:
Methyl β-D-galactopyranoside, nitrophenyl α -L-fucopyranoside, 0.1M potassium phosphate buffer at pH 8, dimethyl sulfoxide and α-L-fucosidase from *Corynebacterium* were incubated at 37°C for 16 hours [100].
A yield of 2-O- α –L-fucopyranosyl-β-D-galactopyranoside of 25% was reached which correlated to a purified amount of 8,5 mg by activated carbon column chromatography [100].
When α-L-fucosidase from *Corynebacterium* and nitrophenyl α -L-fucopyranoside were incubated with acceptor galactose instead of Methyl β-D-galactopyranoside in as to the rest same reaction mix for 12 hours and at 37°C, the product was 2-O- α -L-Fucopyranosyl-D-galactose [100].
A yield of 18% was reached which correlates to 11.4 mg purified product via carbon column chromatography [100].
An *α* -L-fucosidase from *Ampullaria* [100] showed a preference for the formation of α1,6-linkages since it was possible to convert substrate Methyl β-D-galactopyranoside to Methyl 6-O-α-L-fucopyrunosyl-β-D-galactopyranoside.
A reaction mix consisting of Methyl β-D-galactopyranoside, nitrophenyl α -L-fucopyranoside, 0.1M acetate buffer at pH 5.0, dimethyl sulfoxide and *α* -L-Fucosidase from *Ampullaria* (0.2 unit) was incubated for 16 h at 37°C [100].
A yield of 14% was reached which correlated to 4,9 mg purified product by activated carbon column chromatography [100].
Yields of fucosylated products by either *α* -L-fucosydases from *Corynebacterium* sp, *Aspergillus niger* or *Ampullaria* are compared and summarized in table 14 [100]:

Table 14 Production of fucosylated disaccharides via different α-L-fucosidases adapted from Ajisaka and Shirakabe [100]

Acceptor	Origin of enzyme	Linkage	Yield (%)
B-D-GalOMe	Corynebacterium sp.	α-L-(1-2)	25
D-Gal	Corynebacterium sp.	α-L-(1-2)	18
D-Glc	Aspergillus niger	α-L-(1-3)	61
D-GlcNAc	Aspergillus niger	α-L-(1-3)	58
B-D-GalOMe	Ampullaria	α-L-(1-6)	14

It was demonstrated that the mentioned syntheses could be extended to a larger scale. About 1 g of 2-acetamido-2deoxy-3-O-α-L- fucopyranosyl-D-glucose could be obtained by the α-L-fucosidase of *Aspergillus niger from* a large-bore, activated-carbon column (7 x 80 cm) [100].

Example 2: In vitro synthesis of fucosyl-N-acetylglucosamines by 2 different α -L-fucosidases of Lactobacillus casei

As described in example 1 the use of α-L-fucosidases from *Penicillium multicolour* [101] and *Aspergillus niger* [100] did not lead to the synthesis of α-L-1,6 linkages by fucosylation.
An α -L-fucosidase of *Lactobacillus casei* called AlfC [102] in contrast was able to synthesize an α-L-1,6 linkage similar to those of *Ampullaria* [100] as was demonstrated in a study by Diaz et al. [102].
A product called fucosyl- α -1,6-*N*-acetylglucosamine could be obtained in a 56% yield [102].
Another α -L-fucosidase of *Lactobacillus casei* called AlfB was responsible for an α-L-1,6 linkage in the formation of fucosyl-α-1,3-*N*-acetylglucosamine in a 23% yield [102]
The large differences in yields of both enzymes corresponded to a 7.7-fold higher transglycosylation/hydrolysis ratio for AlfC than for AlfB as illustrated in table 15 [102]:

Table 15 Activities of transglycosylation and hydrolysis of AlfB and AlfC adapted from Díaz et al [102]

Enzyme	Transglycosylation (µmol/min/mg protein)	Hydrolysis (µmol/min/mg protein)	V_{trans}/V_{hyd}[a]
AlfB	0,95	1,50	0,63
AlfC	12,79	2,63	4,86

[a]Transglycosylation/hydrolysis (V_{trans}/V_{hyd}) ratios were calculated from the initial rates of formation of transglycosylation products over the initial rates of hydrolysis

Both enzymes were isolated from *Lactobacillus casei* first, expressed with 6(His)-tags in *Escherichia coli* as well as purified to near homogeneity before they were used for the in vitro experiments [102].

Both reaction mixtures consisted of the following substances:

50 mM donor p-nitrophenyl-α-L-fucopyranoside (pNP-fuc), 200 mM acceptor N-acetylglucosamine (GlcNAc), 50 U/ml AlfB or 100 U/ml AlfC whereby 1U means the amount of enzyme required to release 1 mol of pNP in 1 hour [102].

The amount of AlfC used for the reaction mix was twice as high as those of AlfB in order to reach the maximum yields of disaccharide since AlfC shows a significant lower stability in the reaction mix and is inactivated after a 60 minute reaction at 42°C [102].

Figure 28 compares both enzymes with regard to stability in a reaction with p-nitrophenyl-α-L-fucopyranoside (pNP-fuc) under the following conditions [102]:

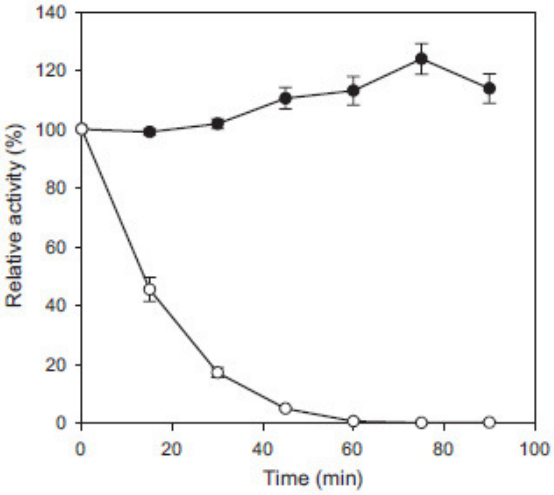


Figure 28 Stabilities of AlfB (symbolized as black circles) and AlfC (symbolized as white circles) adapted from Díaz et al. [102]

Both enzymes were separately incubated in a 100 mM sodium phosphate buffer and p-nitrophenyl-α-L-fucopyranoside (pNP-fuc) at pH 7,0 and at a temperature of 42°C.
The data for each enzyme apparent from table are mean values and were determined from 3 independent reactions under the same conditions.
Standard deviations are given by error bars and the initial activities are described as activities of 100 % [102].
It was possible to avoid byproducts other than the hydrolyzed fucose and pNP by the use of the mentioned reaction mixes and conditions [102].

Example 3: In vitro synthesis of 3`-fucosyllactose and α-L-fucosyl-N-acetyllactosamines by fucosidases from porcine liver and Alcaligenes sp.

In a study by Murata et al. [103] it was reported that an α-L-fucosidase from porcine liver was able to synthesize α -L-Fuc-(1.2)-β-D-Gal-(1.4)-D-GlcNAc (product 1) as well as its isomers α -L-Fuc-(1.3)- β -D-Gal-(1.4)-D-GlcNAc (product 2) and α -L-Fuc-(1.6)- β -D-Gal-(1.4)-D-GlcNAc (product 3) with donor p-nitrophenyl α -L-fucopyranoside and acceptor β -D-Gal-(1.4)-D-GlcNAc (LacNAc).
α -L-Fuc-(1.2)-β-D-Gal-(1.4)-D-GlcNAc (product 1) is called 2`-O-a-L-fucosyl-N-acetyllactosamine and is present in the H antigen structure.
The reactions catalyzed by α-L-fucosidase from porcine liver are illustrated in figure 29 adapted from Murata et al. [103]:

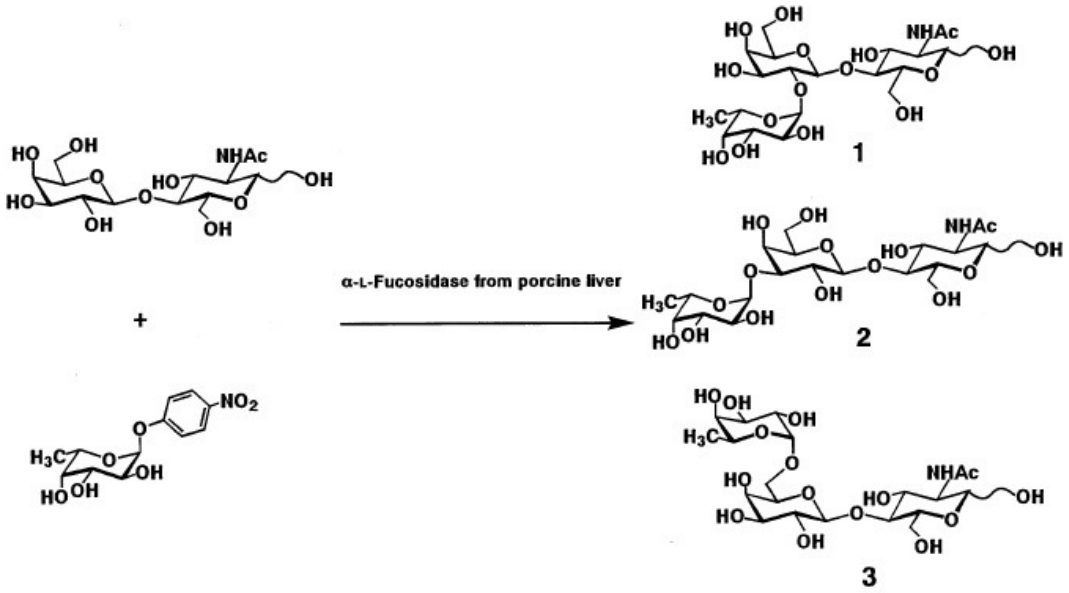


Figure 29 Reaction mechanism of α-L-fucosidase from porcine liver adapted from Murata et al. [103]. Number 1 represents α -L-Fuc-(1.2)-β-D-Gal-(1.4)-D-GlcNAc (product 1), number 2 represents α -L-Fuc-(1.3)- β -D-Gal-(1.4)-D-GlcNAc (product 2) and number 3 represents α -L-Fuc-(1.6)- β -D-Gal-(1.4)-D-GlcNAc (product 3)

The presence of the α -L-Fuc-(1.3)-D-Gal linkage has been reported as part of higher homologues of human milk oligosaccharides (HMO) [104].
As donor p-nitrophenyl α -L-fucopyranoside was used.
Products were obtained from a reaction mix containing Fucα-*p*NP, LacNAc and 50 mM potassium phosphate buffer at pH 4.8 with MeOH as well as partially purified α -L-fucosidase from porcine liver (0.45 U) [103]. The maximal product yield respectively total amount of the three products was obtained after a 24 hour reaction at 40 °C as can be seen in figure 30 adapted from Murata et al. [103]:

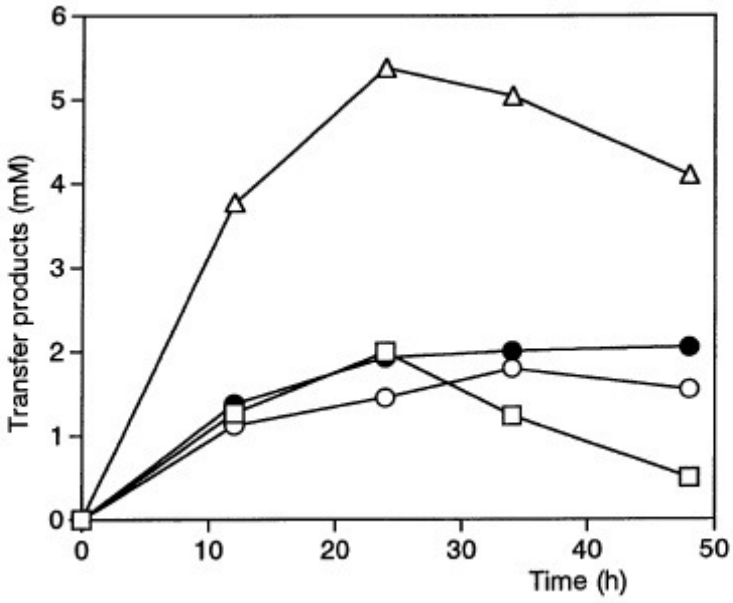


Figure 30 Synthesis of 2`-fucosyl-N-acetyllactosamine and isomers by α-L-fucosidase from porcine liver adapted from Murata et al. [103]. Black circles represent α -L-Fuc-(1.2)-β-D-Gal-(1.4)-D-GlcNAc (product 1), white circles represent α -L-Fuc-(1.3)- β -D-Gal-(1.4)-D-GlcNAc (product 2) and white squares represent α -L-Fuc-(1.6)- β -D-Gal-(1.4)-D-GlcNAc (product 3)

The enzyme was able to produce all three trisaccharides (products 1-3) in 13% overall yield and in the ratio product 1: product 2: product 3 = 40:37:23 which correlated to the purified amounts from the reaction mix via an activated charcoal–Celite column of 6.8 mg α -L-Fuc-(1.2)-β-D-Gal-(1.4)-D-GlcNAc (product 1), 6.4 mg α -L-Fuc-(1.3)- β -D-Gal-(1.4)-D-GlcNAc (product 2) as well as 3.9 mg α -L-Fuc-(1.6)- β -D-Gal-(1.4)-D-GlcNAc (product 3) [103]. Only low regioselectivity was observed, because fucosylation took place without discrimination at the O-2, O-3 and O-6 positions of the Gal moiety as is illustrated in figure 3. Therefore product 1 was obtained in a low yield of 5% [103].

No fucosylated product was observed when lactose instead of LacNAc was tested as an acceptor which indicates that the porcine liver α -L-fucosidase might recognize differences in the acceptor structure [103].

In the same study by Murata et al. [103] fucosylating activity of α -L-fucosidase from *Alcaligenes* sp. was reported to be responsible for the regioselective production of α (1.3)-linked oligosaccharide α -L-Fuc-(1.3)- β -D-Gal-(1.4)-D-GlcNAc (product 2) in 54 % yield and 3`-fucosyllactose (product 4) in 34% yield with β -D-Gal-(1.4)-D-GlcNAc and lactose as acceptor, respectively.

The reactions catalyzed by α -L-fucosidase from *Alcaligenes* sp. are illustrated in figure 31 adapted from Murata et al. [103]:

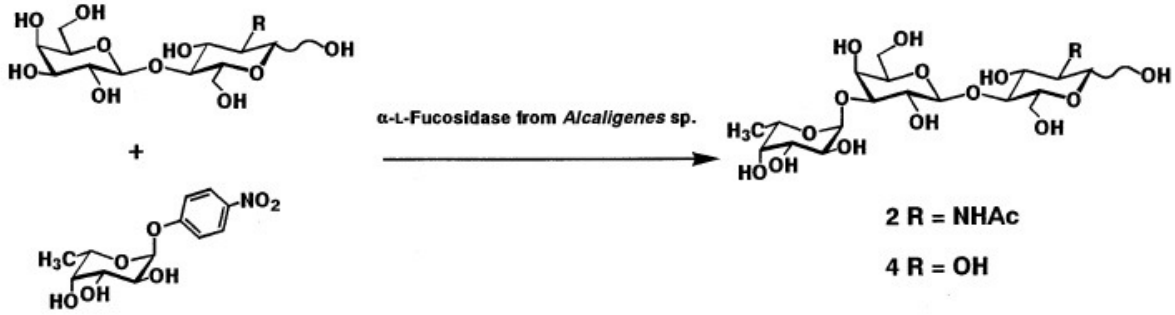


Figure 31 Synthesis of 3`-fucosyllactose (product 4) from lactose as acceptor and Fucα-*p*NP as donor adapted from Murata et al. [103]

The 54 % yield of α -L-Fuc-(1.3)- β -D-Gal-(1.4)-D-GlcNAc (product 2) correlated to an amount of 60 mg which was purified via HPLC with a charcoal–Celite chromatography [103]. The chromatogram showed that only one transfer product was formed after the entire reaction in the following reaction mix:

LacNAc, 20 mM potassium phosphate buffer at pH 7, α –Lfucosidase from *Alcaligenes* sp. (0.6 U) and Fuc α –*p*NP were incubated for 24 h at 50 °C [103].

The 34% yield of 3`-fucosyllactose (product 4) correlated to an amount of 35,2 mg which was purified via an activated charcoal–Celite column after reaction in the following reaction mix [103]:

Fuc α –*p*NP, lactose, 20 mM potassium phosphate buffer at pH 7 and α –Lfucosidase from *Alcaligenes* sp. (0.6 U) were mixed together in order to obtain 3`-fucosyllactose [103].

In addition to the synthesis of α -L-Fuc-(1.3)- β -D-Gal-(1.4)-D-GlcNAc (product 2) it was also demonstrated that 3`-fucosyllactose (product 4) could be produced as the sole transfer product [103].

Figure 32 shows the yields obtained by fucosylation of α -L-fucosidase from *Alcaligenes* sp. and porcine liver [103]:

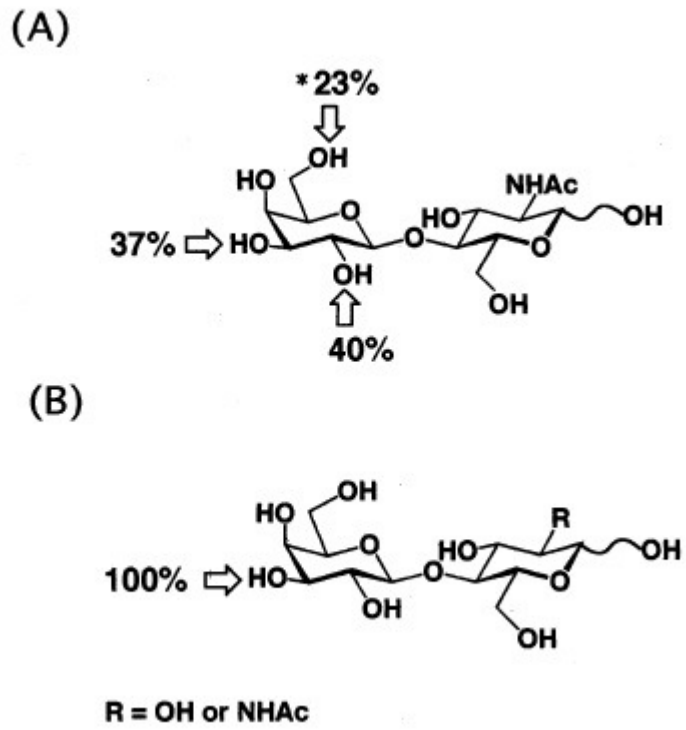


Figure 32 Percentages of α-L-fucosyl-disaccharides synthesized by porcine liver fucosidase (A) and fucosidase of *Alcaligenes* sp. (B) adapted from Murata et al. [103]. The numbers show the individual transglycosylation as compared with the total

According to Baer et al. [105] also other acceptors such as β-D-Gal-(1.4)- β -D-GlcNAcOMe, β -D-Gal-(1.4)- β -D-GlcOMe, and β -D-Gal-(1.3)- β -D-GlcOMe could be used for highly regioselective transglycosylation to the 3-position of the Gal moiety with α-L-fucosidase from *Alcaligenes* sp. and Fuc*α*pNP as donor.

The methyl group at the β anomeric position of the acceptors may enable the regioselective synthesis of transfer products fucosylated at the O-3` position of the galactose-containing disaccharide acceptors by α –L-fucosidase from *Alcaligenes* sp. but not by the porcine liver fucosidase [105].

Regioselective productions by α –Lfucosidase from *Alcaligenes* sp. are compared in table 16 [105]:

Table 16 Product yields by *Alcaligenes* sp in dependence on regioselectivity adapted from Baer et al. [105]

Acceptor	Substrate[a] (%, w/v)	Product	Yield[b] (%)
Galβ1-4Glc	7,3	Fucα1-3 Galβ1-4Glc	34
Galβ1-4Glcβ-OMe	7,3	Fucα1-3 Galβ1-4Glcβ-OMe	42
Galβ1-3Glcβ-OMe	7,4	Fucα1-3 Galβ1-3Glcβ-OMe	67
Galβ1-4GlcNAc	10,3	Fucα1-3 Galβ1-4GlcNAc	54
Galβ1-4GlcNAcβ-OMe	10,3	Fucα1-3 Galβ1-4GlcNAcβ-OMe	67

[a] Total substrate concentration ; [b] Based on the donor added

It should be mentioned that α -L-fucosidase from *Alcaligenes* sp. shows a hydrolyzing activity on 3`-fucosyllactose and 2`-fucosyllactose.
A hydrolizing rate of 77,5 was determined for 3`-fucosyllactose [105].
Since 3`-fucosyllactose was obtained after reaction it can be assumed that the enzymes fucosylating activity on lactose respectively hydrolysing activity of the donor Fuc α –*p*NP are higher than its hydrolysing activity on 3`-fucosyllactose [105].
It could be demonstrated that both, α –L-fucosidase from *Alcaligenes* sp. as well as from porcine liver are convenient for the synthesis of fucosylated human milk oligosaccharide structures whereas those from *Alcaligenes* sp. showed a better regioselectivity in order to produce α -L-Fuc-(1.3)- β -D-Gal-(1.4)-D-GlcNAc (product 2) [103].

Example 4: Conversion of α-L-fucosidase of Thermotoga maritima into an α-L-transfucosidase by directed evolution

α-L-fucosidase of *Thermotoga maritima* is able to slightly transfer α fucosyl residue from α *p*NP-fucoside donor to *p*NP-fucoside through a so called self-condensation with α -1,3 regioselectivity at yields of 10,3% or to *p*NP-galactoside through a so called transglycosylation with α -1,2 regioselectivity at yields of 7% as can be seen in table 18 [95]:
Because it was apparent that natural α-L-fucosidase of *Thermotoga maritima* was not convenient for an appropriate synthesis of fucosylated oligosaccharides at higher yields since

the yield of transglycosylation products did not exceed 30%, directed evolution was used to construct mutants in order to reduce hydrolysing but to achieve higher transglycosylating activity with product yields higher than 60%.
Thereby hydrolysing activity always refers to the hydrolysis of transglycosylated product and not to the release of pNP from *p*NP-fucoside.
Following strategy was used in order to generate and identify positive, more active mutants: Mutations were first introduced through random mutagenesis by a Taq PCR (Error Prone PCR) of gene *TM0306* of α-L fucosidase from *Thermotoga maritima* which led to a mutation rate of 3-4 misincorporated base pairs per 1000 base pairs and an averaged substitution of 2 amino acids per fucosidase enzyme.
The PCR fragments were cloned into a pET21a vector and were amplified via PCR with the aid of primers including a His_6 codon whose expression enabled purifying through Thin Layer Chromatography (TLC).
The His_6 codon- *TM0306*-fusion constructs were cloned into expression vector pBTac 2 expressed in *Escherichia coli* XL1 blue cells and selected with regard to hydrolysis strength first [95].
Escherichia coli cells harbouring fucosidases with lower hydrolysing activity represented pale blue colonies and were further selected via screening of their transglycosylating activity on *p*NP-αFuc (self-condensation) as well as on acceptor *p*NP-αGal and phenyl lactobiose (α-D-Gal(1,3)- α-D-Glc-Ophenyl) with *p*NP-αFuc as donor [95].
Analysis by TLC indicated that three clones respectively mutants called B3, B12 and B36 from first generation showed a higher transglycosylating activity compared to the wild type. After a TLC based screening process it was suggested that four mutations, namely Y237H and Y267F of clone B36, T264A of clone B12 and L322P of clone B3 were responsible for improving transglycosylation [95].
Two of each of the three mutants and all three mutants (B3, B12 and B36) which showed a higher transglycosylating activity compared to the wild type were recombined at the described mutated positions in order to improve transfucosylating activity again.
Recombination between two mutants resulted in the formation of clones C67 with mutations T264A/L322P derived from clones B12 and B3, M3 with mutations Y237H/Y267F/L322P derived from B36 and B3, N16 with mutations *M210V*/Y237H/T264A derived from B36 and B12 as well as R8 with mutations *M210V*/Y237H/T264A/Y267F derived from B36 and B12 [95].

Recombination between all three mutants resulted in the formation of clones P25 with mutations *G226S*/ Y237H/T264A/L322P, S1 with mutations *M210V*/Y237H/T264A/ Y267F/L322P, C2 with mutations T264A/Y267F/L322P as well as D2 with mutations *G226S*/T264A/Y267F/L322P.
Mutations *M210V* and *G226S* were introduced inadvertently by Error Prone PCR.
Additional mutants called S1 and D2 were prepared to define the effect of these mutations [95].
Kinetic parameters concerning hydrolysis and transglycosylation were determined via capillary electrophoresis from a reaction mix incubated at 40 °C and pH5,5 consisting of *p*NP-αFuc as donor (10 mM), *p*NP-*α*Gal as acceptor (10mM) and 1 unit enzyme.
Further on kinetic parameters concerning hydrolysis and transglycosylation were determined at 70°C with the same reagents but a pH of 5,5.
Kinetic parameters are depicted in table 17 [95]:

Table 17 Kinetic parameters of wild type and mutated fucosidases at a temperature of 40°C and a pH of 5,5 as well as a temperature of 70°C and a pH of 5 adapted from Osanjo et al. [95]

Mutant	Temp. (°C)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/ K_m
WT	40	5,1±0,2	0,020±0,003	$2,6 \times 10^5$
	70	35±1	0,079±0,007	$4,4 \times 10^5$
B3	40	15±0,5	0,13±0,01	$1,2 \times 10^5$
	70	198±11	0,28±0,03	$7,1 \times 10^5$
B12	40	15±0,4	0,065±0,006	$2,3 \times 10^5$
	70	169±18	0,07±0,01	$2,5 \times 10^6$

B36	40	15±0,8	0,09±0,01	1,7 x 10^5
	70	164±4	0,10±0,01	1,6 x 10^6
C67	40	3,6±0,1	0,19±0,02	1,9 x 10^4
	70	50±4	0,25±0,06	2,0 x 10^5
M3	40	14,8±0,6	0,29±0,05	5,1 x 10^4
	70	96±9	0,23±0,04	4,2 x 10^5
N16	40	20±0,5	0,10±0,01	2,0 x 10^5
	70	65±5	0,06±0,01	1,1 x 10^6
R8	40	4,3±0,1	0,09±0,01	4,7 x 10^4
	70	56±4	0,14±0,02	4,0 x 10^5
P25	40	2,7±0,1	0,08±0,01	3,3 x 10^4
	70	39±1	0,10±0,01	3,9 x 10^5
S1	40	0,73±0,02	0,14±0,01	5,2 x 10^3
	70	20±0,7	0,25±0,02	8,0 x 10^4
C2	40	0,50±0,6	0,06±0,01	8,3 x 10^3
	70	8,2±0,4	0,31±0,04	2,7 x 10^4
D2	40	0,48±0,01	0,09±0,01	5,3 x 10^3
	70	6,4±0,5	0,30±0,06	2,1 x 10^4

According to table 17 it was demonstrated that mutants from the first round of mutagenesis showed higher k_{cat} values that were not expected since the mutants were screened for lower hydrolytic activity values -ascribed to the pale blue colonies from XL1 blue cells compared to the wild-type [95].

But in addition K_m values were also higher and as a result lower specificity constants K_{cat} / K_m were calculated which corresponded to the lower hydrolytic activity obtained from these mutants.

This lower hydrolytic activity could be explained by a better stabilization and lifetime of the covalent enzyme-glycosyl intermediate respectively α-L-fucosidase-pNP-αFuc intermediate resulting in a higher K_m value than those of the wild type enzyme [95].

There was no correlation between k_{cat} values for *p*NP-αFuc (self-condensation) and transglycosylating activity because k_{cat} values were obtained from both, hydrolytic activity on *p*NP-αFuc as well as the self-condensation reaction [95].

For mutants B3, B12, B36, M3, N16 a higher k_{cat} was calculated compared to the wild type which indicated an inhibitory effect of a higher amount of *p*NP-*α*Gal on enzyme activity of the wild type.

Hydrolytic activity for each enzyme refers to the hydrolysis of transglycosylated product and was determined by abstracting transglcosylating activity from activity with regard to the release of pNP from pNP-α-Fuc (overall activity).
In table 18 activities are summarized [95]:

Table 18 Hydrolytic, transglcosylating and overall activity as well as yields of the wild type and mutants adapted from Osanjo et al. [95]

Mutant	Transglycosylation of pNP Fuc(1-3)Fuc µmol/(min mg)	Transglycosylation of pNP Fuc(1-2)Gal µmol/(min mg)	Hydrolysis µmol (min mg)	Overall activity µmol/(min·mg)	V_{trans} to V_{hyd}	Maximum yield (%) of pNP Fuc(1-3)Fuc	Maximum yield (%) of pNP Fuc(1-2)Gal
WT	198±19	303±110	2954±213	3523±198	0,2	10,3	7
B3	445±41	2612±67	1897±60	5031±73	1,7	12,4	41
B12	568±42	3923±210	1855±230	6603±173	2,6	13.7	42
B36	792±141	2560±191	2694±344	6823±190	1,5	16.9	28
C67	35±7	1545±135	392±100	2010±200	4,0	5,6	55
M3	300±71	3238±288	868±17	4560±364	4,1	6,1	56
N16	622±109	4350±184	2495±67	7467±345	2,0	9,5	40
R8	146±20	2743±148	1330±96	4287±224	2,2	7,2	44
P25	26±3	1650±160	246±32	2020±140	6,8	5,4	62
S1	3,1±0,2	351±21	56±10	401±22	6,3	4,4	55
C2	1,3±0,1	161±4	29±5	189±2	5,5	4,6	59
D2	1,3±0,2	144±24	33±11	177±6	4,4	5,8	65

Transglycosylation and hydrolysis activity of the wild type and mutants were measured at pH 5 and 40°C in the presence of pNP- α-Fuc (10mM) and pNP-β-Gal (10mM).
Activity of transglycosylation could be measured after transglycosylation products pNP-Fuc(1,3)Fuc (self condensation) and pNP-Fuc(1,2)Gal were separated by capillary electrophoresis [95].

Mutants B3, B12 and B36 showed a transglycosylation to hydrolysis (T/H) ratio approximately more than 10 times higher than those of the wild type as well as a higher overall activity which correlated to higher k_{cat} values.
Through recombination between mutants B3, B12 or B36 this ratio could be increased significantly by up to 30 times according to the wild type whereas the overall activity changed in a smaller range which led to a slower hydrolysing but higher transglycosylating activity. Thus mutants like C67 from the second generation for instance hydrolysed less substrate *p*NP-αFuc indeed but a lot less product *p*NP α-L-Fuc*p*-(1,2)-*β*-D-Galp so that a maximum yield of 55% was observed [95].
The highest formation of *p*NP α-L-Fuc*p*-(1,2)-*β*-D-Galp with a yield of 65% could be observed with mutant D2 followed by P25 with 62% as can be seen in table 18 and figure 33.
Figure 33 shows the formation of *p*NP α-L-Fuc*p*-(1,2)-*β*-D-Galp by wild type and mutants B3, B12, B36 (all first generation) and mutants R8, P25 and D2 (all second generation) over a period of 800 minutes [95]:

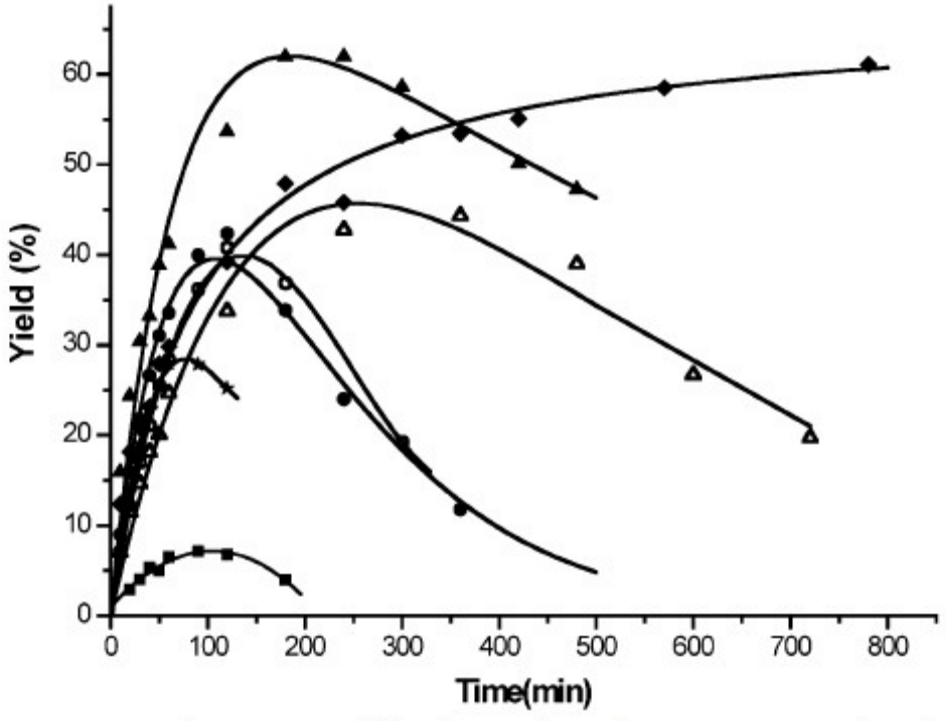


Figure 33 Production of pNP α-L-Fucp-(1-2)-β-D-Galp by wild type and mutants adapted from Osanjo et al. [95]. Black squares represent wild type, white circles represent mutant B3, black circles mutant B12, black stars mutant B36, white triangles mutant R8, black triangles mutant P25 and black rhombi mutant D2

According to figure 33 it is obvious that reaction time of mutant D2 was significantly longer to reach almost the same maximum product yield of P25 which refers to the much lower transglycosylating and overall activity obtained from D2 as can be seen in table 18 [95].
In general it was obvious that recombinations of two mutants from the first generation resulting in mutants C67, M3 and R8 showed higher overall activities than those mutants including all three mutations (S1, C2, D2).
For latter ones the overall activity was only 5-10% compared with the wild type while mutants M3 and R8 showed overall activities higher than those of the wild type enzyme.
For mutants such as P25 or D2 from the second generation the increase in the formation of pNP α-L-Fucp-(1,2)-β-D-Galp was committed with a decrease of pNP α-L-Fucp-(1,2)- α-D-Galp (self condensation) and thus a higher selectivity compared to the wild type which preferred the synthesis of pNP α-L-Fucp-(1,2)-β-D-Galp by a yield of only 10%.
In figure 34 this co-development of transglycosylation/hydrolysis activity ratio and specificity is shown [95]:

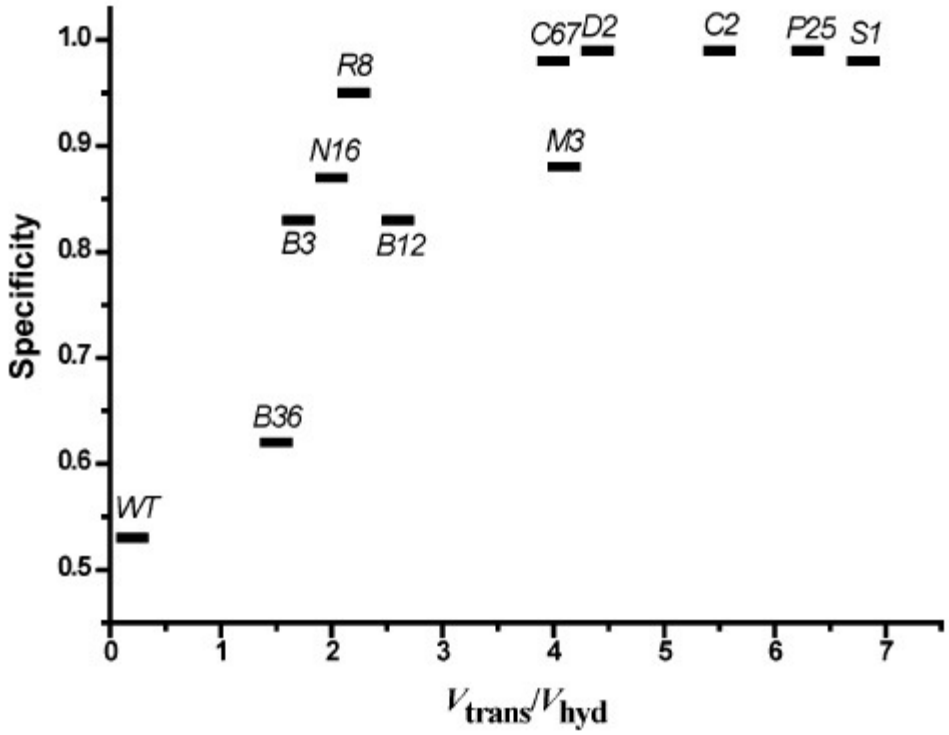


Figure 34 Transglycosylation to hydrolisis ratio at the initial rate adapted from Osanjo et al. [95]. The y axis includes the specificity of transglycosylation respectively the ratio of pNP α-L-Fucp-(1-2)-β-D-Galp to the maximum concentration of all transglycosylation products

It was demonstrated that several screening processes and mutations on wild type enzyme α-L-fucosidase of *Thermotoga maritima* could positively influence the enzymes specificity and product yield in order to synthesize higher amounts of transglycosylating product pNP α-L-Fucp-(1,2)-β-D-Galp by yields above 60% and minimize self-condensation [95].
It could not be accurately determined if the three mutations T264A, Y267F and L322P of mutants of the second generation supported improving the yield of Fucp-(1,2)-β-D- Galp through an accumulating effect but it was assumed that mainly mutation T264A played an important role [95].
Through mutation T264A in the second amino acid shell of the active site from mutants P25 or D2, the hydrogen bond between T264 and R254 was avoided and a significant reorientation of the R254 side chain was induced which enabled the formation of a new hydrogen bond with higher specificity to acceptor *p*NP-*α*Gal than to *p*NP-αFuc and an efficient production of pNP α-L-Fucp-(1,2)-β-D-Galp hence.
The structural change in the active site by mutation T264A is illustrated in figure 35 adapted from Osanjo et al. [95]:

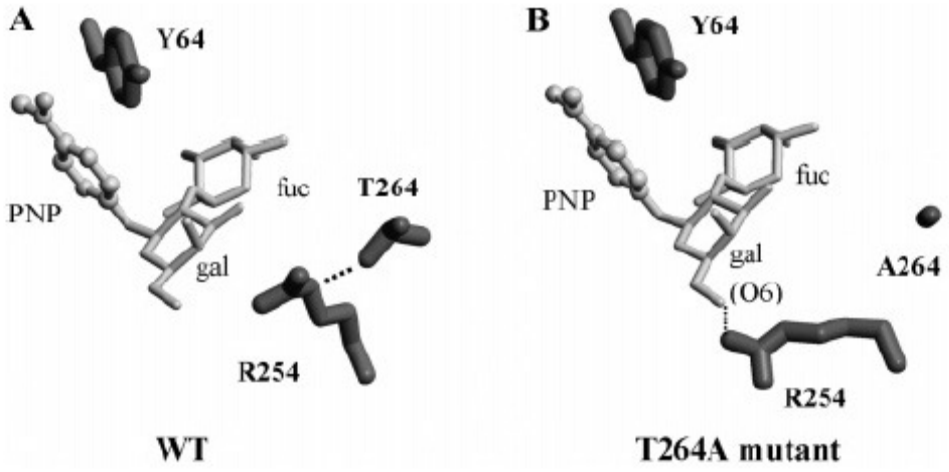


Figure 35 The structural change in the active site by mutation T264A adapted from Osanjo et al. [95]

To sum up mainly a mutation of T264A might be essential to improve α-L-fucosidase of *Thermotoga maritima* in order to efficiently synthesize 2`-fucosylated disaccharides such as pNP α-L-Fucp-(1,2)-β-D-Galp [95].

Usage of an 1,2-α-L-Fucosynthase in the enzymatic synthesis of human milk oligosaccharides (HMO)

1,2- *α* -L-Fucosidase (AfcA) of *Bifidobacterium bifidum* [93] belongs to the inverting *α* -glycosidases that hydrolyse Fucα1,2Gal linkages.
Amino acids N423 and D766 of the catalytic domain of 1,2- *α* -L-Fucosidase (AfcA) are important in the hydrolysis of 2`-fucosyllactose because they are essential for the activation of an attacking water molecule whereas N421 is involved in maintaining the water molecule and the side-chain of E566 which represents a general acid in their proper orientations.
Thus asparagine-423 (N423) activated by a neighbouring aspartic acid-766 (D766) acts as a base while asparagine-421 (N421) is responsible for the formation of a hydrogen bond to an attacking water molecule and positions the side-chain of the acid residue glutamic acid-566 (E566) for hydrolysis.
Figure 36 illustrates this catalytic mechanism [93]:

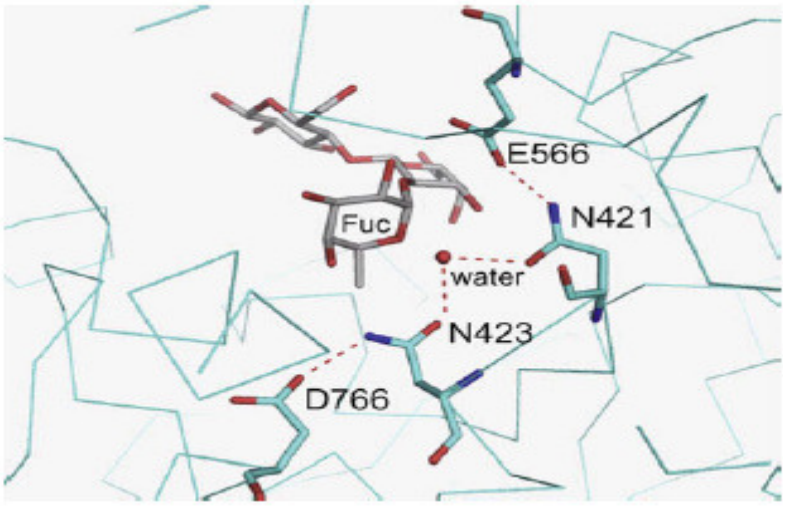


Figure 36 Catalytic site of AfcA with important residues involved in catalysis adapted from Wada et al. [93]

The acknowledge about this mechanism played a key role in the development of a fucosynthase by Wada et al. [93] which derived from the inverting 1,2-α-L-Fucosidase AfcA of *Bifidobacterium bifidum*.

It was able to generate 2`-fucosylated carbohydrate structures such as 2`-fucosyllactose by introducing α 1,2-fucosyl linkages on galactose residues at the non-reducing ends of sugar chains with the aid of the donor fucosyl fluoride.
The fucosyl fluoride with the opposite anomeric configuration compared to the enzyme mimics and decreases formation of the fucosyl-enzyme intermediate involved in the hydrolysis of the desired fucosylated product.
Therefore transglycosylation of the substrate is possible [93].
One advantage of the fucosynthase based in vitro system is that this donor can be produced at low costs in contrast to GDP-L-fucose which is essential for in vitro (and in vivo) syntheses with fucosyltransferases.
Two strategies have been suggested for the construction of a glycosynthase from an inverting enzyme in order to inhibit hydrolysis of product(s) and promote transglycosylation.
The first one is based on a replacement of a general base residue with a non-catalytic residue, the second on a replacement of a residue holding a non-polar residue with a catalytic water molecule. Both could be convenient in order to avoid hydrolytic activity while the function of the general acid residue remains which acts in turn as a base and abstracts a proton from an incoming acceptor [93].
A maximum product yield of 2`-fucosyllactose could be obtained by the former approach by the replacement of asparagine aspartic acid-766 to glycin which resulted in mutant D766G as can be seen in the following chromatogram received from HPLC [93]:

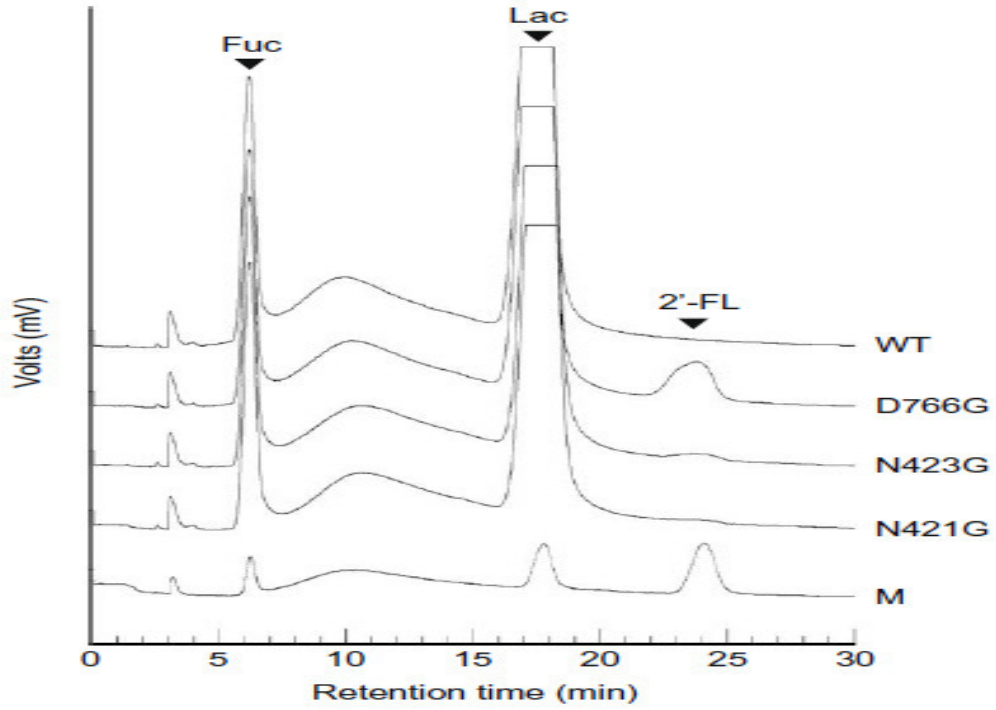


Figure 37 Chromatogram of glycosynthase mutants and wild type adapted from Wada et al. [93]. Fuc stands for fucose, Lac for lactose and 2`-FL for 2`-fucosyllactose

Since aspartic acid-766 (D766) was replaced through glycine it was not able to activate asparagine-423 (N423) acting as a base and hydrolysis of 2`-fucosyllactose was avoided.
In addition to mutant D766G, glycine mutants N421G and N423G were constructed in order to avoid hydrolysis on 2`-fucosyllactose but achieve effective formation of it.
But in contrast to mutant D766G those mutants were not able to produce high amounts of 2`-fucosyllactose.
The rising concentration of 2`-fucosyllactose over a period of 2 hours is depicted in figure 38 adapted from Wada et al. [93]:

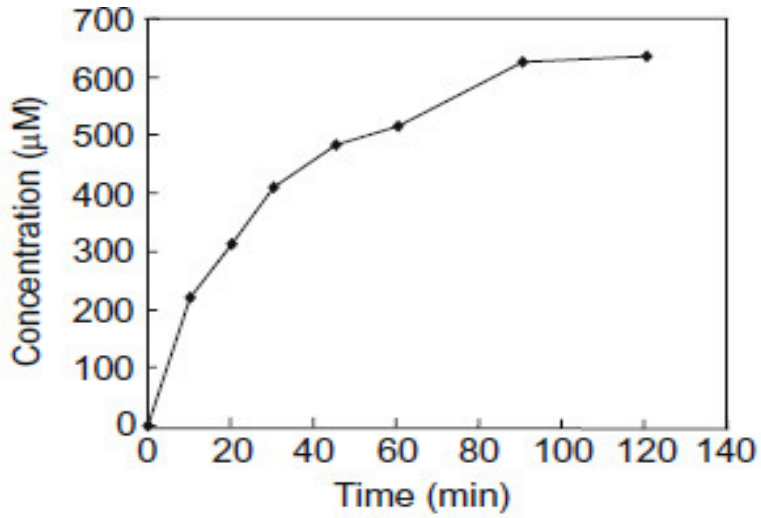


Figure 38 Concentration profile of 2`-fucosyllactose by a reaction based on mutant D766G adapted from Wada et al [93]

More than 600 µM 2`-fucosyllactose respectively approximately 3 mg could be produced from acceptor lactose and donor fucosyllactose after a 2 hour reaction with 70 µg of mutant D766G from 100 µL of the following reaction mix:
To obtain 2`-fucosyllactose yields each mutant inclusively mutant D766G was incubated with 50 mM MOPS buffer at pH 7.0, 10 mM fucosyl-fluoride as donor and 30 mM lactose as acceptor for 1 hour and at a temperature of 30 °C.
A yield of only 6% 2`-fucosyllactose compared to the added fucosyl fluoride was estimated. The low yield and non linearity of the reaction rate observed over time could be explained either because of a decreasing concentration of fucosyl fluoride by its spontaneous hydrolysis or an inhibition of the synthetic reaction by higher amounts of L-fucose caused by the decomposition of fucosyl fluoride [93].

Usage of α-L-fucosyltransferases in the enzymatic synthesis of human milk oligosaccharides (HMO) or core structures thereof

Although FucT2 from *Helicobacter pylori* [13;81] is probably the most frequent used fucosyltransferase in the production of human milk oligosaccharide 2`-fucosyllactose, fucosyltransferases from other bacterial origins or mammals inclusive humans were determined and tested on substrate specificity in vitro as for example α1,2-FucT WbsJ of *Escherichia coli* O128:B12 or human FucT-III.
The following examples describe the usage of α-L-fucosyltransferases in the synthesis of fucosylated human milk oligosaccharides (HMO) or core structures thereof.
Thereby the α-L-fucosyltransferases were either used in their natural occurring structure or genetically engineered.

Example 1: In vitro synthesis of α-L-fucosyl-N-acetyllactosamines by combination of α-L-glycosidase and α-L-fucosyltransferase

In a patent by Nilsson et al. [20] it was shown that a process based on a combination of hydrolases in the form of glycosidases as well as glycosyltransferases is responsible for an efficient synthesis of glycosylated compounds.
In the case of α-L-fucosyltransferase as glycosyltransferase the production of α-L-fucosyl-N-acetyllactosamines that are fundamental human milk oligosaccharide building blocks is possible if donor GDP fucose is present.
In this invention a glycosidase is responsible for the catalysis of a shorter oligosaccharide which shows a lower regioselectivity and serves as a substrate for the glycosidase respectively intermediate for the formation of the fucosylated oligosaccharide with a higher regioselectivity.
Thereby glycosyltransferases like a fucosyltransferase are used for the final formation of this more complex oligosaccharide end product.

With regard to α-L-fucosyltransferase activity different fucosyl-N-acetyllactosamines can be obtained according to the patent by Nilsson et al. [20].
The use of glycosides as acceptors for the glycosidase-catalysed reaction results in a glycoside product called N-acetyllactosamine which can be easy purified since anomerisation of the product glycoside does not occur.
In addition a certain glycosidase might be used for predominant synthesis of various isomers because of its low regioselectivity so that glycosides with different aglycons or configuration can serve as ligand.
The reaction scheme describing this invention is illustrated in figure 39 adapted from Nilsson et al. [20]:

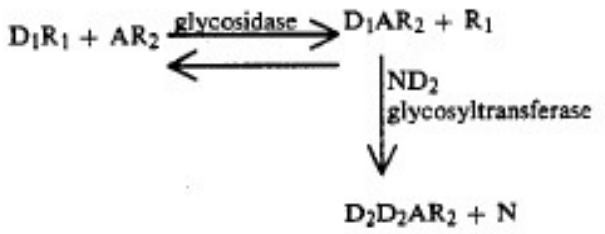


Figure 39: Reaction scheme of combined glycosidase *and* glycosyltransferase adapted from Nilsson et al. [20]

AR_2 represents the acceptor saccharide, D_1R_1 the donor saccharide, D_1AR_2 a glycoside intermediate needed for the formation of the end product (oligosaccharide) which is symbolized as $D_1D_2AR_2$.
R_1 represents a by-product after hydrolysis of D_1R_1 by a glycosidase and ND_2 the second donor saccharide (a nucleotide sugar) needed in synthesis of the end product.
N stands for a certain nucleotide which is obtained during fucosylation by a glycosyltransferase [20].
With this invention the synthesis of different compounds including fucosylated human milk oligosaccharide core structures in form of fucosyl-N-acetyllactosamines might be possible:
First compound Fucαl-2Galβl-3GlcNAcβ-OMe may be produced whereby Fucαl-2Galβl-3GlcNAcβ-R occurs in lacto-N-fucopentaose I and lacto-N-difucopentaose I.

Second compound Fucαl-2Galβ1-4G1cNAcβ-OMe might be synthesized whereby Fucαl-2Galβ1-4G1c is the human milk oligosaccharide called 2`-fucosyllactose and Fucαl-2Galβ1-4G1cNAcβ is called 2`-O-a-L-fucosyl-Nacetyllactosamine and includes H antigen structure. Third a production of Galβl-4(Fucαl-3)GlcNacβ-OMe should be possible whereby Galβl-4(Fucαl-3)GlcNacβ-R occurs in lacto-N-fucopentaose III [20].

Synthesis of Fucαl-2Galβl-3GlcNAcβ-OMe may be achieved through two reaction mixes: Reaction mix number one is prepared in order to produce Galβl-3GlcNAcβ-OMe first. Thereby GlcNAcβ-OMe (the acceptor saccharide AR_2) as well as Gal(β)-OPhNO2-o (the donor saccharide D_1R_1) are resuspended and incubated at 37°C for a period of 4 days in sodium phosphate buffer at pH 6,5, dimethylformamide and β-D-galactosidase from bovine testes with an activity of 2U.

Column chromatography can be used to obtain pure Galβl-3GlcNAcβ-OMe.

A second reaction mix is then prepared to synthesize end product Fucαl-2Galβl-3GlcNAcβ-OMe by mixing Galβl-3GlcNAcβ-OMe as acceptor in 0,1 M MES-CHI at a pH of 6,7, GDP-fucose as donor, α1,2-fucosyltransferase, Triton X-100 as well as albumin and incubating them at 37°C.

Analogous Fucαl-2Galβ1-4G1cNAcβ-OMe may be synthesized but with a β1-4 isomer (Galβl-4GlcNAcβ-OMe) as acceptor.

Furthermore formation of Galβl-4(Fucαl-3)GlcNacβ-OMe should be feasible by this invention by using the same procedure as described for the production of Fucαl-2Galβ1-4G1cNAcβ-OMe but with an α1,3-fucosyltransferase instead of an α1,2-fucosyltransferase [20].

The invention by Nilsson et al. [20] mediates the possibility of an efficient in vitro synthesis of α-L-fucosyl-N-acetyllactosamines based on a combination of glycosidase- as well as fucosyltransferase-activity.

Two separated reactions are essential:

Hydrolysis of a certain donor saccharide concomitant with a glycosylation of a special acceptor saccharide through a glycosidase and its fucosylation with the aid of a fucosyltransferase as illustrated in figure 39.

Example 2: Use of α1,2-fucosyltransferase WbsJ of Escherichia coli O128:B12 as a fusion protein in the in vitro synthesis of 2`-fucosyllactose

Although α1,2-fucosyltransferase WbsJ [14] is classified to the same family as Fut1, Fut2 and FucT2 from *Helicobacter pylori*, it shows only a marginal sequence similarity to them. This sequence similarity is based on three common motifs named motif 1, 2 and 3 that are also found in α1,6-fucosyltransferases and O-fucosyltransferases but not in α1,3-fucosyltransferases which indicates that WbsJ, α1,6-fucosyltransferases and O-fucosyltransferases origin from the same ancestor gene. The motifs might be located adjacent to each other and play important roles in catalytic mechanism [14]. In contrast to other fucosyltransferases such as human Fut1 and Fut2 or *Helicobacter pylori* Fucosyltransferase FucT, WbsJ shows broad acceptor specificity to Gal*β*1,4Fru (lactulose), Gal*β*1,3GalNAc, Gal*β*1,4Man and Gal*β*1,4Glc (lactose) [14] and might therefore be convenient to produce a high variety of fucosylated oligosaccharides and probably 2`-fucosyllactose.
Acceptor respectively substrate specificity as well as relative reactivity of WsbJ with regard to different acceptors are depicted in table 19 [14]:

Table 19 Relative activities of fusion protein GST-WbsJ with various acceptors adapted from Li et al. [14]

Acceptor (10 mM)	Relative activity (%)	Acceptor (10 mM)	Relative activity
Galβ1-4-Glc (lactose)	100±1,4	Gal	35,7±1,1
Galβ1-4-Glcβ-N_3	137±3,7	Galβ-O-Me	68,8±4,0
Galβ1-4-Glcβ-O-ph	84,7±4,3	Galβ1-4GlcNAc	12,4±0,2
Galβ1-4-Glcβ-S-ph	207±9,7	Galβ1-4Fru (lactulose)	380±14,9
Galβ1-4-Glcβ-1-NAc	199±11,2	Galβ1-4Gal	No data
Galα1-4Gal	No data	Galβ1-4Man	162±4,7
Galα1-4Galβ1-4-Glc	No data	Galβ1-3GalNacα-O-Bn (T antigen)	202±6,7
Galα1-3Galβ1-4-Glc	No data	Galβ1-3GalNacα-O-Me (T antigen)	215±5,9
Galα-PNP	No data	GalNacβ1-3Galα1-4Galβ1-4-Glc	No data
Galβ-PNP	30,4±0,7		

Moreover WbsJ might belong to the glycosyltransferase family B which does not require EDTA and divalent metal cations in contrast to glycosyltransferase family A: Addition of them mainly reduced WbsJ activity although low concentrations of kations Mn^{2+} below 5 mM and Mg^{2+} of 10 mM or below have a positive stimulating effect on WbsJ [14].
Figure 40 shows relative activities of WbsJ by addition of EDTA and different metal cations at 10 mM concentrations compared to WbsJ activity without addition [14]:

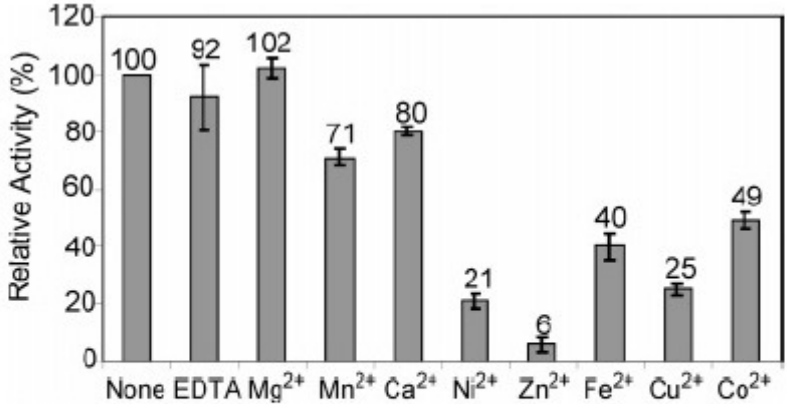


Figure 40 Relative activity of α1,2-fucosyltransferase dependent on EDTA and metal cations adapted from Li et al. [14]. Pure reaction without EDTA or metal cations is described as "None". To all other reactions 10 mM metal cation or EDTA was added

As can be seen in figure 40 only Mg^{2+} has a small positive influence on WbsJ at a concentration of 10 mM whereas 10 mM Mn^{2+} still show a decrease in activity compared to the approach without addition [14].
The fusion protein GST-WbsJ facilitated purification through chromatographic processes in contrast to a His_6 –tag and other fusion constructs but also showed a better stability compared to the free form [14] and thus might be the better alternative in production of fucosylated oligosaccharides such as 2`-fucosyllactose.
The recombinant fusion protein showed a high relative activity of about 100 % when lactose was added which makes it to a potential candidate for 2`-fucosyllactose synthesis although Galβ1,4Fru (lactulose) was identified to be the best acceptor with a reaction rate nearly four times faster than that of lactose [14].
A replacement of the reducing terminal glucose with GlcNAc caused a change to a very poor substrate whereas compounds with a terminal R-Gal did not serve as acceptors at all.

Obviously the C2-OH of glucose in lactose could be important for enzyme recognition since the enzyme activity of LacNAc is only 12.4% compared to that of lactose [14].
The big differences in activity concerning acceptors Galβ1,4Gal (0%) and Galβ1,4Man (162%) may lay in the fact that the C2-OH of Gal is in the equatorial conformation whereas the C2-OH of Man shows the axial conformation [14].
The enzyme was identified as active in an in vitro Fucosyltransferase Activity Assay within pH 5.5- 8.5 and by a temperature of 37°C when lactose as substrate was added.
The highest activity was measured at a pH between 6 and 7.4 probably because of ionization of the catalytic residue which enhances binding affinity and / or stability of the enzyme.
Enzyme activity was strong inhibited by GDP at 1 mM but ATP, ADP, AMP, and L-R-fucose did not influence enzyme activity at the same concentration when different amounts of lactulose as acceptor and a constant amount of GDP-fucose as donor were used. According to kinetic studies WbsJ was competitively inhibited by GDP with an apparent *K*i value of 1.41 mM as can be seen in figure 41 adapted from Li et al. [14]:

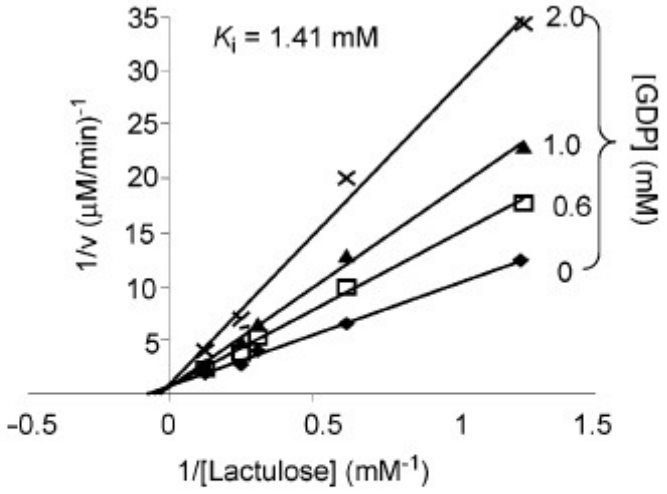


Figure 41 Lactulose dependent inhibition of GDP on the α1,2-fucosyltransferase activity of GST-WbsJ adapted from Li et al. [14]. K_i value of GDP for GST-WbsJ was achieved through variation of lactulose concentration at various GDP concentrations (0, 0,6, 1,0, and 2 mM) and a constant GDP-L-fucose concentration of 0,30 mM

Kinetic parameters of recombinant GST-WbsJ fusion protein mainly corresponded to the measured activity data and were determined by either variation of acceptor concentration of Galβ-O-Me, lactose, and lactulose, respectively, or donor concentration of GDP-L-fucose.

Since lactulose was proofed to be the best acceptor for GST-WbsJ fusion protein it showed the highest apparent V_{max} and k_{cat} value by far compared to the other receptors. V_{max} was about 6 times higher and k_{cat} approximately 3 times higher than those of lactose although the K_m of lactose was half as high as those of lactulose [14].
Galβ-O-Me could not be synthesised efficiently which can be explained through a high K_m and small V_{max} value.
Donor GDP-L-fucose was tested as efficient substrate because it showed the lowest apparent K_m of all tested substances and the second highest apparent V_{max} and k_{cat} after lactulose [14].
Different mutants of α1,2-FucT- glutathione S-transferase (GST) - fusion proteins of *Escherichia coli* O128:B12 were separately expressed by Li et al. [14] in *E. coli* BL21 (DE3) in order to improve the enzymes activity. Mutations of the *wbsJ* gene which encodes the α1,2-FucT in *Escherichia coli* O128:B12 or more precise of the conserved motif HxRRxD which was named motif 1 and is found in the α1,2-FucT family and the α 1,6-FucT family were based on two-step PCR respectively Quik Change site-directed mutagenesis [14].
This motive could stand for an important key factor concerning binding of donor GDP-L-fucose and enzyme catalysis because of its presence in the α1,2-FucT family and the α 1,6-FucT family and might be an important tool for mutations to change enzyme activity [14].
Substitution of H152 by either Ala (A) or Arg (R) decreased the enzyme activity to 19.2% or 11.5% of the wild type and mutation R154A showed a marginal activity of 3% compared with the wild type WbsJ. Replacement of R154 by the positively charged residue Lys (K) was able to restore activity to 12.0% which indicates the positive charge as well as the size of the side chain of R154 reduces R1,2- FucT activity. Also mutation of R155A caused a strong reduction in activity of 15.4%. The conservative mutation of R155K by contrast restored the activity to 31.1% which demonstrates that R155 might be less essential than R154. Moreover in motif HxRRxD, the last conserved residue D157 was replaced to Ala (A), Glu (E) or Asn (N) but all three mutants D157A, D157E and D157N could show only low activity values of 4.1%, 6.8% and 5.3%, respectively. Replacement with a similar acidic residue through mutation D175E caused no remarkable difference in activity compared with D157A.
R154 and D157 may play more important roles than other residues concerning to the results obtained in the study by Li et al. [14].
It must be mentioned that all mutants were tested on lactulose and not lactose and therefore it is difficult to say if these mutations have also an effect on 2`-fucosyllactose synthesis but it should not be excluded since lactose was tested as an efficient substrate for WbsJ too.

Compared to the wild type fusion protein WbsJ-GST no mutant was able to show a higher activity as shown in figure 42 adapted from Li et al. [14]:

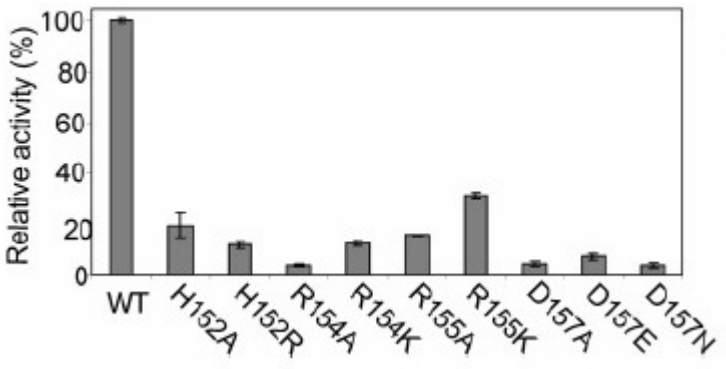


Figure 42 Relative activities of *Escherichia coli* wild type and mutants adapted from Li et al. [14]

The kinetic parameters of seven mutants (H152A, H152R, R154K, R155A and R155K) that possessed higher enzyme activities were determined by using the same method as those of the non mutated fusion protein WbsJ [14].

Mutant H152A gave an apparent *K*m value for donor GDP-fucose which was twice as large as of the value for H152R (Table 4). This result indicated the need for a positive residue at this position. The change of H152 to R also resulted in a significant increase of the *K*m value for the acceptor, probably because the bulky side chain of Arg interferes with binding of the acceptor, leading to weaker binding affinity [14].

Mutants R155A and R155K showed similar *K*m values for the acceptor (76.0 mM and 64.8 mM, respectively).

However, mutant R155A, which eliminates the positive charge, yielded a K_m value for the donor substrate three times greater than that of mutant R155K. These results suggest that preservation of the positive charge at residue R155 is more important than the length of the side chain for the binding of GDP-L-fucose as well as enzyme function. This data is consistent with the results of the specific enzyme activities of the mutants [14].

It could be demonstrated that mutations in the motif 1 of WbsJ could not improve V_{max} compared to the wild type WbsJ-GST and reduced it significantly whereas the K_m value of all mutants increased dramatically as can be seen in table 20 [14].

Table 20 Kinetics of wild type and GST-WbsJ mutants adapted from Li et al. [14]

Enzyme	Apparent K_m of acceptor lactulose (mM)	Apparent V_{max} of acceptor lactulose (µM/min/mg)	Apparent K_m of donor GDP-fucose (mM)	Apparent V_{max} of donor GDP-fucose (µM/min/mg)
Wild type	14,4±1,1	9,1±1,4	0,122±0,005	12,8±0,2
H152A	55,3±9,3	2,8±0,3	0,865±0,150	5,6±0,7
H152R	96,7±24,3	5,7±0,8	0,462±0,140	3,5±19,1
R154K	108±27,6	5,5±0,9	0,575±0,070	8,4±0,7
R155A	76,0±7,3	5,5±0,9	1,997±0,640	12,1±3,4
R155K	64,8±6,5	1,9±0,1	0,628±0,271	5,2±1,4

Although mutations of motif 1 did not improve kinetic parameters and activity compared with the wild type fusion protein WbsJ, they could be still an efficient tool to enhance them by alternative mutations that were not tested in this experiment.
Furthermore motifs 2 and 3 were not changed but could play important key factors in synthesis too.

Example 3:In vitro and in vivo formation of Le^a and Le^x epitopes by natural human fucosyltransferases and chimera thereof

Human fucosyltransferase III (FucT III) is a fucosyltransferase with α1-4 fucosyltransferase activity encoded by the Lewis (*Le*) gene. In vivo it catalyzes the transfer of GDP-activated fucose in α1-4-linkage to subterminal *N*-acetylglucosamine on type 1 chain Gal(βl,3)GlcNAc called N-lactobiose which results in the formation of Le^a epitopes.
This enzyme shows also an α1-3 fucosyltransferase activity on type 2 chain Gal(βl,4)GlcNAc called N-acetyllactosamine responsible for synthesis of Le^x epitopes although its specificity for type 2 chains is smaller [33].
Fucosyltransferases FucT III as well as FucT .V show both, an α1,3 fucosylating activity on type 2 chains and an α1,4 fucosylating activity on type 1 chains [19;33;50;56;57].
In a study by Legault et al. [106] it was demonstrated that in vivo and in vitro fucosylation of type I acceptor substrates such as Lacto-N-biose (Gal*β*1-3GlcNAc) by human α(1,3)-fucosyltransferases Fuc TIII and Fuc TV probably strongly depend on a short, 61-amino acid-

long peptide segment which represents a hypervariable region and includes subdomains 4 and 5 of the NH2 terminus of the Golgi lumenal domain proximal to the enzymes sequence-constant COOH-terminal domains. This region may be involved in the acceptor substrate recognition through as few as eleven residues and might be significantly altered in human α(1,3)-fucosyltransferase Fuc TVI so that fucosylation of type I oligosaccharides is not feasible [106].

Moreover it can be assumed that acceptor substrate specificity can be indirectly determined by subdomain-dependent proteolytic effects [106].

An efficient fucosylation of type I substrates was possible by FucTIII and Fuc TV that include unique subdomain residues whereas fucosylation with Fuc TVI was not feasible likely because of alternative, 21 nonidentical amino acid residues found in this region.

Important structural differences of Fuc TV compared to Fuc TVI and Fuc TIII that are suggested to be responsible for the loss of type 1 utilization are based on an arginine residue instead of a tryptophan residue at position 110, two arginine residues instead of a pair of proline residues at positions 122 and 126, a tryptophan residue in contrast to glutamine (Fuc TIII) or arginine (Fuc TV) at position 143 and last but not least a lysine residue which replaces a glutamic acid residue at position 146 [106].

First recombinant fucosyltransferase chimeras were constructed by exchanging the nearly identical COOH termini of Fuc TIII and Fuc TVI with each other and then expressed in COS-7 cells in order to test substrate utilization whereby four chimeras were found to be informative for the influence of COOH termini on substrate specificity.

In two of these chimeras called Fuc TC1 and Fuc TC3, the exchange took place between amino acid at postion 301 of Fuc TIII or 300 of Fuc TVI and the COOH terminus of the alternative gene resulting in a 60-amino acid-long peptide segment in Fuc TIII respectively 59-residue long peptide in Fuc TVI.

They showed differences in three amino acid positions and the presence (Fuc TIII) or absence (Fuc TVI.) of a valine residue at position 353 [106].

The other two chimeras called Fuc TC2 and Fuc TC4 were formed after the entire COOH-terminal regions were exchanged whereby Fuc TC2 included the COOH terminus of Fuc TVI and Fuc TC2 the COOH terminus of Fuc TIII [106].

It could be demonstrated that COS-7 cells transfected with chimeras Fuc TC3 and Fuc TC4 which did not loose the NH2-terminal segment of Fuc TVI and included portions of the COOH terminus of Fuc TIII were able to produce the Le(x) and sLe(x) epitopes but no Le(a) or sLe(a) epitopes that both show type 1 structure [106].

COS-7 cells harbouring chimeras Fuc TC1 or Fuc TC2 which maintain the NH2-terminal segment of Fuc TIII and portions of the COOH terminus of Fuc TVI by contrast were able to synthesize the Le(x), Le(a), and sLe(a) epitopes but no sLe(x) antigen [106].
Through in vitro experiments it could be demonstrated that wild type Fuc TIII showed similar Michaelis-Menten constants for type II substrate N-acetyllactosamine and type I substrate lacto-Nbiose with 12.7 and 8,1 mM, respectively, although the enzymes relative specific activity was significantly higher on the type I substrate.
Contrary, Fuc TVI showed a big difference in K_m values for the type II acceptor (4.9 mM) and the type I acceptor (greater than 50 mM) and its features were similar to those of Fuc TC4. It exhibited a significantly higher relative specific activity toward the type II substrate [106].
To develop the dependence of the substrate specificities on the hypervariable region recombinant α1,3-fucosyltransferase chimeras derived from Fuc TIII and Fuc TVI were constructed via site-directed respectively cassette mutagenesis.
After dividing the hypervariable region into five distinct subdomains that were different in Fuc TIII and Fuc TVI, single subdomain exchanges between subdomains 4 or 5 among others as well as multiple subdomain exchanges between subdomains 4 and 5, 1,2 and 3 as well as 3,4 and 5 in the NH2-terminal hypervariable region were performed in Fuc TIII and Fuc TVI in order to express α1,3-fucosyltransferase chimeras [106].
Each of the five Fuc TIII chimeras with a single Fuc TVI subdomain was able to synthesize those four cell surface antigens in vivo that could be also produced by the wild type Fuc TIII. Thereby two of these chimeras called Fuc TC8 and Fuc TC9 that showed substitutions in subdomain 4 or 5, respectively, showed a reduction in the formation of Le(a) and sLe(a) antigens and a relative increase in the production of the type II-based epitopes Le(x) and sLe(x).
This observations corresponded to a ratio of Le(a) positive cells (29.8%) to Le(x) positive cells (37.2%) for wild type Fuc TIII of 0.80 and a ratio of 0.19 when Fuc-TC8 was expressed whereby 9.1% Le(a) positive and 47.9% Le(x) cells could be determined. Furthermore a ratio of 0.29 was calculated for Fuc-TC9 because 10.2% Lea positive as well as 35.4% Lex positive cells were determined. Similar ratios were observed for the sialylated forms of these antigens [106].
Further chimeras were constructed via multiple subdomain exchanges.
For instance Chimera Fuc TC15 which included subdomains 1, 2 and 3 from Fuc TVI after transposition into the wild type Fuc TIII sequence was convenient for the formations of Le(x), sLe(x), Le(a), and sLe(a) determinants similar to those of the wild type Fuc TIII [106].

By contrast the complementary chimera Fuc TC17 which included domains 1, 2, and 3 from Fuc TIII after transposition into wild type Fuc TVI could only utilize type II acceptor substrates to produce Le(x) and sLe(x) as was also observed for the wild type Fuc TVI [106]. Therefore transposition of domains 1, 2, and 3 together may not efficiently influence type I acceptor substrate utilization whereas transposition of both subdomains 4 and 5 significantly influence type I substrate recognition [106].

The exchange of subdomains 3, 4, and 5 in Fuc TIII with those of Fuc TVI resulted in the fucosyltransferase chimera called Fuc TC16 which could convert type II substrates to Le(x) or sLe(x) determinants.

Conversely the transposition of subdomains 3, 4, and 5 from Fuc TIII in Fuc TVI resulted in the fucosyltransferase chimera called Fuc TC18 which expressed all four epitopes in vivo but at reduced absolute levels (Le^x, 8.1% positive; sLe^x, 3.0% positive; Le^a, 4.8% positive; and sLe^a, 4.0% positive) with regard to levels observed from wild type Fuc TIII [106].

According to α(1,3)-Fucosyltransferase activity assays the specific activity of this chimera was

significantly lower than those of Fuc TIII or Fuc TVI on type I and type II acceptor substrates lacto-N-biose I and N-acetyllactosamine where Km values were higher than 50 mM for these substrates [106].

Sole transposition of subdomain 3 which varies only by the presence (Fuc TVI) or absence (Fuc TIII) of a consensus sequence for asparagine-linked glycosylation had no visible effect on acceptor substrate utilization as was suggested from transfection analyses of the mutants Fuc TIII and Fuc TVI chimeras called Fuc TC7 and Fuc TC12, respectively.

Thus only exchange of both subdomains 4 and 5 might have a positive effect on type 1 and type 2 utilization which can be seen on chimeric α(1,3)-Fucosyltransferase Fuc-T19 and Fuc-T20 that showed similar features to those fucosyltransferases that were constructed through the exchange of subdomains 3, 4, and 5 [106].

Fuc TC19 including subdomains 4 and 5 from Fuc TVI after transposition into Fuc TIII utilized type II acceptor substrates with approximately the same efficiency of the wild type enzyme Fuc TVI but no type I acceptors which corresponded to the in vitro measurements of a high relative specific activity and a low K_m on the type II acceptor of 1.1 mM and a high K_m for the type I acceptor which was upper 50 mM.

By contrast complementary chimera Fuc TC20 constructed via transposition of subdomains 4 and 5 from Fuc TIII into Fuc TVI converted neutral and sialylated type I molecules, as well as neutral type II molecules in vivo.

Thereby Lea (6.3%positive cells), sLea (3.3% positive cells), and Lex (5.4% positive cells) antigens could be synthesized [106].

Interestingly, in vitro a low specific activity toward both, type I and type II acceptors could be determined which referred to *Km* values above 50 mM although Fuc TC20 utilized the type I acceptor substrate much more efficiently compared to the type II acceptor as was also observed for the wild type and Fuc TC18.

In contrast to Fuc TC18 which could produce all four epitopes Fuc TC20 could not synthesize sialyl Lewis x but the other three fucosylated epitopes which could be explained because of an absence of an asparagine-linked glycosylation site within subdomain 3 in Fuc TC18 resulting in an intact Fuc TVI scaffold [106].

The most important chimeras determined in this study are summarized and compared with regard to their kinetic parameters in table 21 [106]:

Table 21 Kinetic parameters of fucosyltransferase chimeras adapted from Legault et al. [106]

Enzyme	Apparent K_m of acceptor LacNAc (mM) (N-acetyllactosamine)	Apparent K_m of acceptor LNB-I (mM) (lacto-N-biose I)	Specific activity ratio, LacNAc/LNB-I
Fuc- TIII	8,1	12,7	0,12
Fuc- TVI	4,9	>50	2610
Fuc- TC2	>50	>50	0,03
Fuc- TC4	5,8	>50	3646
Fuc- TC18	>50	>50	0,05
Fuc- TC19	1,1	>50	189
Fuc- TC20	>50	>50	0,04

According to Legault et al. [106] these results demonstrate the influence of sequences within subdomain four and five of Fuc TIII (respectively Fuc TVI) on the type I acceptor (respectively type 2 acceptor) recognition and utilization.

In addition the COOH terminus of Fuc TVI may have a positive effect on type 2 acceptor recognition and utilization as can be seen on chimera Fuc TC4 whereas the COOH terminus of Fuc TIII might influence the synthesis of type 1 products as demonstrated on chimera Fuc TC2.

Usage of human GDP-mannose 4,6-dehydratase and FX protein in the in vitro synthesis of GDP-Fucose, an intermediate in the synthesis of fucosylated human milk oligosaccharides (HMO)

Fucose synthetase or synonymous GDP-4-keto-6-deoxy-D-mannose epimerase/reductase (GMER) is a bifunctional enzyme which catalyzes both an epimerization at C3 and C5 and an NADPH-dependent reduction on C4 of GDP-4-keto-6-deoxymannose which leads to GDP-L-fucose production [107].

Due to enzyme fold and its main structural features fucose synthetase can be classified in the reductase–epimerase–dehydrogenase (RED) enzyme homology superfamily.

Protein fucose synthetase encoded by the *wcaG* gene and other enzymes catalyzing either NAD(P)H-dependent reduction reactions or nucleoside diphosphate sugar epimerization reactions show a high similarity to the human FX protein.

The human FX protein shows a more than 50 % identity to the protein fucose synthetase encoded by *wcaG*, formerly known as *yefb* , whose gene maps in a region of *Escherichia coli* chromosome coding for enzymes involved in synthesis and utilization of GDP-D-mannose. Thus the FX protein which was purified from human erythrocytes could be identified to perform the same conversion of GDP–4-keto-6-deoxy-D- mannose to GDP–L-fucose [23;24].

By the use of recombinant human GDP-mannose 4,6-dehydratase and FX protein (GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase) that were first expressed in *Escherichia coli* BL21(DE3) as fusion proteins separately and then purified through successive chromatography it could be demonstrated that the two proteins alone are sufficient to convert GDP-mannose to GDP-L-fucose in an in vitro production system:

Sequential incubation of GDP-mannose with human dehydratase and NADP+ followed by human epimerase-reductase and NADPH converts GDP-mannose to GDP-L-fucose.

The human and *Escherichia coli* dehydrogenases are compared in table 22 [107]:

Table 22 Kinetic parameters of human and *Escherichia coli* GDP-mannose-4,6-dehydratase adapted from Sullivan et al. [107]

	Apparent K_m (µM)	V_{max} (µmol/min/mg)	IC_{50} 0f GDP (µM)	IC_{50} 0f GDP.fucose (µM)	Stimulation by NADPH	50% stimulation by NADPH (µM)
Human	80	0,11	225	75	4,5 fold	2
Escherichia coli	260	0,73	400	10	2,3 fold	15

In contrast to the human and *Escherichia coli* dehydratases that show a strict cofactor preference for NADP+ over NAD+ it was determined that the human and *Escherichia coli* epimerase-reductase can utilize NADPH as well as NADH, although NADPH is used more efficiently [107].

The *Escherichia coli* enzyme has a significantly higher K_m for GDP-mannose than the human enzyme but also a significantly higher V_{max}.

Both dehydratases are stimulated by NADPH at micromolar concentrations, although this co-factor is not involved in catalysis directly and shows an inhibition by GDP-L-fucose with IC50 values lower than the IC50 values for inhibition by GDP. As a reason GDP-L-fucose concentration might play an essential role in regulation respectively feedback inhibition of dehydratase activity and in turn 2`-fucoyllactose production [107].

In the study by Sullivan et al. [107] an efficient alternative to *Escherichia coli* enzymes was identified in human enzymes in order to synthesize GDP-fucose which represents an important intermediate in the synthesis of human milk oligosaccharides (HMO).

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