Synthesis of *N*-acetyllactosamine and *N*-acetyllactosamine-based bioactives

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Abstract

N-Acetyllactosamine (LacNAc), or more specifically β-D-galactopyranosyl-1,4-*N*-acetyl-D-glucosamine, is a unique acyl-amino sugar and a key structural unit in human milk oligosaccharides, an antigen component of many glycoproteins and an antiviral active component for the development of effective drugs against viruses. LacNAc is useful itself and as a basic building block for producing various bioactive oligosaccharides, notably because this synthesis may be used to add value to dairy lactose. Despite a significant amount of information in the literature on the benefits, structures and types of different LacNAc-derived oligosaccharides, knowledge about their effective synthesis for large scale production is still in its infancy. This work provides a comprehensive analysis of existing production strategies of LacNAc and important LacNAc-based structures, including sialylated LacNAc, as well as poly- and oligo-LacNAc. We conclude that direct extraction from milk is too complex, while chemical synthesis is also impractical at an industrial scale. Microbial routes have application when multiple step reactions are needed but the major route to large scale biochemical production will likely lie with enzymatic routes, particularly those using β-galactosidases (for LacNAc synthesis), sialidases (for sialylated LacNAc synthesis) and β-*N*-acetylhexosaminidases (for oligo-LacNAc synthesis). Glycosyltransferases, especially for the biosynthesis of extended complex LacNAc structures, could also play a major role in the future. In these cases, immobilization of the enzyme can increase stability and reduce cost. Processing parameters, such as substrate concentration and purity, acceptor to donor ratio, water activity and temperature can affect product selectivity and yield. More work is needed to optimize these reaction parameters and in the development of robust, thermally stable enzymes to facilitate commercial production of these important bioactive substances.

Abbreviations:

β3GalT	β1-3 galactosyltransferase
β4GalT	β1-4 galactosyltransferase
CMP-NeuNAc	cytidine-5'-monophosphate-5-*N*-acetylneuraminic acid
DS'LNT	α2-6-linked disialyllacto-*N*-tetraose
DSLNnT	α2-6-linked disialyllacto-*N*-neotetraose
EPEC	enteropathogenic *Escherichia coli*
GalNAc	*N*-acetylgalactosamine
GfCoV	Guinea fowl coronavirus
GlcNAc	*N*-acetylglucosamine
GLP-1	glucagon-like peptide 1
HEp-2	human epithelial type 2 cells
iGnT	β1-3 *N*-acetylglucosaminyltransferase
IGnT	β1-6 *N*-acetylglucosaminyltransferase
*i*LNO	*iso*-Lacto-*N*-octaose
LacNAc	*N*-acetyllactosamine
LacNAc-ITag	imidazolium-tagged LacNAc
LacY	β-galactoside permease
lacZ	β-galactosidase gene
lgtA	β1-3 *N*-acetylglucosaminyltransferase gene
lgtB	β1-4 galactosyltransferase gene
LND	Lacto-*N*-decaose
LNH	Lacto-*N*-hexaose
LNnD	Lacto-*N*-neo-decaose
LNnH	Lacto-*N*-neo-hexaose
LNnO	Lacto-*N*-neo-octaose
LNnT	Lacto-*N*-neotetraose
LNO	Lacto-*N*-octaose
LNT	Lacto-*N*-tetraose
ManNAc	*N*-acetylmannosamine
MERS-CoV	Middle East respiratory syndrome coronavirus
Neu5Ac	*N*-acetylneuraminic acid
*o*NPG	*ortho*-nitrophenyl-β-galactoside
*p*LNH	*para*-Lacto-*N*-hexaose
*p*LNO	*para*-Lacto-*N*-octaose
*p*NPG	*para*-nitrophenyl-β-galactoside
poly-LacNAc	poly-*N*-acetyllactosamine
SLN	sialyl-*N*-acetyllactosamine
SPIONs	superparamagnetic iron oxide nanoparticles
UDP-Gal	uridine-5'-diphosphate galactose
UDP-Glc	uridine-5'-diphosphate glucose

INTRODUCTION

To date, over 150 different human milk oligosaccharide (HMO) structures have been discovered.[1] The focus of this review is *N*-acetyllactosamine (LacNAc; *N*-acetyl-4-*O*-(β-D-galactopyranosyl)-D-glucosamine or β-D-galactopyranosyl-1,4-*N*-acetyl-D-glucosamine; Figure 1), which is a core structure in many of these HMOs, including lacto-*N*-neotetraose (LNnT; LacNAc-β1-3-Lactose),a series of lacto-*N*-octaose HMOs, sialyllacto-*N*-neotetraose c (LSTc) and monofucosyllacto-*N*-hexaose (MFLNH).[2-3] LNnT (Figure 1) is of particular note, as it is recommended for the manufacture of safe, well-tolerated, age-appropriate infant formula[4] and is available in more than 30 countries within such products.[5-6] Commercial-scale synthesis of these HMOs, however, is limited.[5]

(Figure 1)

Some more complex HMO structures can be obtained by elongating other core structures or precursors.[7-8] Poly-*N*-acetyllactosamine (poly-LacNAc; Figure 1) structures are not HMOs but have been investigated in a number of studies, due to their essential biological roles in cell-cell interactions, tumor progression and immune response.[9-11] These structures are found on the surface of a range of animal cells including rat, rabbit, human erythrocytes and human leukocytes.[10] Poly-LacNAc structures can be synthesized enzymatically using either LacNAc and LNnT.[12-13] As LacNAc and poly-LacNAc can be used as specific lectin target molecules and bind to the galectin carbohydrate recognition site, they are also useful biomarkers for the detection of cancer cells.[11, 14-19]

LacNAc can also act as a precursor for many other biological compounds, namely glycolipids,[20-21] glycoproteins[20, 22] and some red blood cell components, including the Lewis and

ABO blood group antigens.[23-24] The P1 and P^k antigens are examples of LacNAc-containing bioactives (Figure 1), which are recognized as potential inhibitors of pathogens and toxins, including *Shigella dysenteriae*, *Escherichia coli* O157, *E. coli* O104, *Streptococcus suis*, *Pseudomonas aeruginosa* and HIV virus.[25] Interestingly, it has also been demonstrated that *Lactobacillus casei* can efficiently consume LacNAc, resulting in a stronger induction of the lac genes compared to lactose.[23] As one of the probiotics in the human intestinal tract, *L. casei* can decrease symptoms of anxiety or depression and treat diarrhea,[26-27] making the interaction between LacNAc and *L. casei* potentially valuable.

LacNAc has a number of potentially beneficial physiological effects and appears to work through a variety of mechanisms. Hyland et al.[28] showed that LacNAc glycosides conjugated to BSA (LacNAc–BSA) or gold nanoparticles (LacNAc-Au) inhibited the localized adherence of enteropathogenic *Escherichia coli* (EPEC) to human epithelial type 2 (HEp-2) cells. LacNAc may also assist drug stability, for example glucagon-like peptide 1 (GLP-1), which is proposed for the treatment of type 2 diabetes.[29-30] The *in vivo* use of this hormone is restricted due to its physical instability and aggregation, short half-life in plasma and inactivation by proteolytic enzymes.[31-32] Ueda et al.[33] demonstrated that the addition of LacNAc and α2-6-sialyl-*N*-acetyllactosamine (6'sialyl LacNAc or 6'-SLN) to GLP-1 (Figure 1) could enhance this proteolytic stability and thus the *in vivo* blood glucose-lowering activity of GLP-1. Given the broad potential of glycosylation to increase stability, to enhance activity, to alter tissue specificity, reduce toxicity and alter blood brain permeability,[34-36] there may be much broader potential for LacNAc across a range of pharmaceutical applications.

LacNAc may also have potential use in Magnetic Resonance Imaging (MRI). This technique is widely used as a powerful diagnostic method to produce tissue images. Gadolinium

(Gd^{3+}) complexes are commonly used as MRI contrast agents to enhance the quality of these images but the release of Gd^{3+} in the human body is of major concern given Gd^{3+} toxicity.[37-38] Recently, superparamagnetic iron oxide nanoparticles (SPIONs) coated with LacNAc (Figure 1) have been developed as an alternative MRI contrast agent, which is more biocompatible with human body fluids.[39]

The possible application of LacNAc-based molecules in antiviral drugs has also been emphasized in several studies. Influenza viruses are constantly changing and the evolution of mutant viruses spread through human-to-human contact can have fatal consequences.[40-41] Currently, the number of humans and poultry vaccinated each year is insufficient for the prevention of an outbreak.[42-43] Wang et al.[44] proposed that antiviral drugs based on sialylated LacNAc molecules (3'-SLN and 6'-SLN; Figure 1) may be effective against all mutant viruses, as these molecules are the cell receptors for the hemagglutinin binding of influenza viruses to the epithelium within the human respiratory tract. In a recent study by Kocabiyik et al.[45] a new class of anti-influenza macromolecules based on a cyclodextrin core and 6'-SLN or 3'-SLN terminals were described. They showed that these antiviral drugs can suppress several human or avian influenza strains *in vitro,* as well as a 2009 pandemic influenza strain *ex vivo* and *in vivo*. Likewise, Sriwilaijaroen et al.[46] developed a new drug based on 6'-SLN (6'-SLN-lipo PGA; Figure 1) for eradication of human influenza. This mechanism may also be useful across species, as it has been shown that Guinea fowl coronavirus (GfCoV) can bind to di-*N*-acetyllactosamine and glycans capped with α2-6-linked sialic acids.[47] A further example is the Middle East respiratory syndrome coronavirus (MERS-CoV), as no vaccine or specific treatment is currently available for this virus, which results in an acute respiratory infection in humans following attachment of the virus to human cells via a spike protein. Park et al.[48] recently investigated the attachment of MERS-CoV

spike protein to sialylated receptors, including 3'-SLN and 6'-SLN as potential viral inhibitors, indicating a further potential need for sialylated molecules.

Another potential use of LacNAc-based antiviral drugs is for diarrhoeal diseases, which are commonly caused by rotaviruses among infants and children. These viruses infect most children at least once by the age of five.[49] Symptoms of rotaviruses range from mild to potentially life-threatening.[49] According to the Australian Department of Health, no specific treatment for infected individuals of any age has been developed yet for rotaviruses and they remain highly contagious pathogens. The rotavirus vaccines that are currently available can only be given to young children, whereas older people and those with weakened immune systems are also susceptible to rotavirus infection. It has been demonstrated that poly-LacNAc structures can bind to rotaviruses, acting as antiviral inhibitors,[50-51] suggesting a possible preventive effect of these molecules in public health responses.

Despite the very broad potential use of LacNAc-based bioactives described above, no commercial LacNAc product has been reported to date, beyond LNnT. Given this potential and the lack of a comprehensive review on the available production approaches, we present this review to compare and analyze different production methods for these important *N*-acetyllactosmine and *N*-acetyllactosamine-based molecules. Although chromatography and/or membrane separation techniques have been developed to extract these compounds from milk,[13, 52-53] these purification processes are too complicated for scale-up in their current form owing to low concentrations of the target LacNAc molecule in the eluent stream and the possible presence of other saccharides with a similar structure to that of the target molecule in milk. For example, de Moura Bell et al.[53] showed that the concentration of lactose in bovine colostrum (22 g l^{-1}) is 110 times higher than the concentration of oligosaccharides (0.2 g l^{-1}). Furthermore, although a final oligosaccharide mixture

with an 85% purity was achieved after several purification steps, this mixture only contained 17% 6'-SLN as the LacNAc-based saccharide.[53] Thus, synthetic approaches towards target *N*-acetyllactosamine related structures have attracted widespread attention.

The review commences with a discussion of the potential for chemical production. The focus, however, is on biochemical production and on the enzymatic synthesis of LacNAc and its derivatives, in which lactose can act as a readily available and cheap donor or acceptor. Process and reaction engineering strategies for optimal yield are described, followed by approaches to the purification of these molecules.

CHEMICAL SYNTHESIS

LacNAc can be produced through a series of chemical reactions.[54-55] These methods are, however, not suitable for industrial production due to the use of toxic reagents, such as hydrogen cyanide, as well as the production of many intermediates; low stereoselectivity and the presence of epimers; as well as low overall yields. In a study by Lattová and Petruš[56] an ozonolysis reaction was employed to produce LacNAc (32% yield) from 3-*O*-β-D-galactopyranosyl-D-arabinose. Likewise, 2-acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-D-mannopyranose (a LacNAc epimer) was obtained with a yield of 15%.

More recently, an improved Heyns re-arrangement method was employed to approach large-scale chemical production of LacNAc from lactulose.[57] Lactulose is commercially produced from lactose.[58] According to this method (Figure 2), lactulose (25 g) was reacted with benzylamine to produce ketosyl amine. Afterwards, the mixture obtained was precipitated from diethyl ether. A rearrangement reaction was then performed to convert ketosyl amine, as a crude yellow solid (35 g), to *N*-benzyl lactosamine, within 2 hours using glacial acetic acid in methanol at room

temperature. In the next step, hydrogenolysis of *N*-benzyl lactosamine to D-lactosamine hydrochloride was conducted using hydrogen in the presence of HCl and palladium hydroxide on carbon ($Pd(OH)_2/C$). The crude D-lactosamine hydrochloride was then *N*-acetylated by adding sodium methoxide and subsequently acetic anhydride to give LacNAc. A total yield of 48% on a 25 g scale was achieved after peracetylation, using acetic anhydride and chromatographic separation. Although commercially available lactulose can be used in this reaction, with relatively higher yields, it requires several reagents and the product isolation is also problematic.

(Figure 2)

More complex chemical routes have also been developed for the synthesis of LacNAc derivatives. Lemieux et al.[59] synthesized a LacNAc derivative, hexa-*O*-acetyl-2-deoxy-2-phthalimido-β-D-lactosyl chloride, from D-lactal hexaacetate via a seven-stage azido-nitration route. In a study by Malet and Hindsgaul[60] 24 LacNAc derivatives carrying carboxymethyl, amidinomethyl, aminoethyl and carbamoylmethyl substituents were chemically generated.

Bandara et al.[61] developed a linear synthetic approach for the chemical synthesis of LNnT as shown in Figure 3. They synthesized a glucosamine thioglycoside precursor (**1**) based on a seven-step process previously developed by Nagorny et al.[62] This precursor was then reacted with fluorenylmethyloxycarbonyl chloride (FmocCl) in the presence of pyridine in CH_2Cl_2 to obtain its Fmoc derivative (**2**) which was subsequently converted to a phosphate donor (**3**) in the presence of dibutyl phosphate, freshly activated molecular sieves (3 Å) in CH_2Cl_2, *N*-iodosuccinimide and triflic acid. A lactose precursor (**4**) was synthesized via the coupling between an orthogonally protected galactose donor and a tetrabenzylated glucose 4-OH acceptor in a series of chemical reactions previously reported.[63-64] A trisaccharide (**5**) was then produced via glycosylation between

the phosphate donor (**3**) and the lactose acceptor (**4**) in the presence of trimethylsilyl trifluoromethanesulfonate. Triethylamine in CH_2Cl_2 was then used to remove the Fmoc protecting group to achieve a trisaccharide acceptor (**6**). The trisaccharide acceptor obtained (**6**) was reacted with a *S*-benzoxazolyl donor (**7**) in the presence of silver trifluoromethanesulfonate to produce a β-linked tetrasaccharide intermediate (**8**)**.** The intermediate (**8**) was deprotonated in two steps including refluxing with $NH_2NH_2–H_2O$ in MeOH followed by acetic anhydride in MeOH treatment. The resulting *N*-acetylated tetrasaccharide (**9**) was treated with 10% palladium on charcoal in wet ethanol to eliminate benzyl groups, resulting in the target LNnT product (**10**) with an overall yield of 57%. Likewise, several poly-LacNAc structures have been synthesized through a complicated series of chemical reactions.[65-70] The many steps required, however, represent a barrier for these processes being commercially competitive.

(Figure 3)

BIOCHEMICAL SYNTHESIS

Biochemical synthesis of LacNAc-derived compounds has attracted a great deal of attention due to a low environmental footprint, the possibility of using low-cost, readily available substrates, operation at mild conditions and high selectivity.[71-72] The production can be designed using either naturally occurring and engineered microorganisms as whole cell biocatalysts (*in vivo* production) or enzymes (*in vitro* production). Biochemical synthesis of LacNAc can use phenyl β-D-galactopyranosides, uridine diphosphate galactose or lactose as donor substrates.[73-74]

The use of cheaper substrates for such biochemical synthesis could facilitate commercial production. Lactose is an abundant and inexpensive substrate for the synthesis of value-added functional food products[75-76] and can be utilized in both *in vivo* and *in vitro* biochemical approaches

for LacNAc synthesis. For comparison, the price of 1 kg of lactose (Sigma L3750) is US$519, while the price of 1 kg of *p*-nitrophenyl β-D-galactopyranoside from the same supplier (Sigma N1252) is US$234,000. Each year large amounts of lactose are generated in whey within the global dairy and cheese industry. Whilst this is increasingly used as a valuable by-product, some is discarded with no practical utilization.[77]

Microbial production

Microorganisms can be viewed as living factories capable of the synthesis of LacNAc-derived molecules. They are particularly useful for multi-step reactions requiring more than one enzyme, as microorganisms can be engineered to contain an enzyme cascade.[78] Furthermore, inherent cofactor regeneration such as ATP regeneration by cells is recognized as one of the main advantages of whole cell biosynthesis.[79] As a case in point, without the need for the addition of any ATP cofactor, genetically modified *Escherichia coli* expressing GlcNAc 2-epimerase and *N*-acetylneuraminic acid (NeuAc) aldolase, produced NeuAc from pyruvate and GlcNAc. NeuAc is the most common form of sialic acid, which can be found in the structure of sialylated oligosaccharides, including sialyl LacNAc.[80]

Endo et al.[81] developed a coupled microbial system containing two recombinant *Escherichia coli* strains and *Corynebacterium ammoniagenes* for the production of LacNAc from UDP-Gal (uridine-5'-diphosphate galactose) (Figure 4(A)). The UDP-Gal production was performed by a recombinant *E. coli* expressing the UDP-Gal biosynthetic genes (*E. coli* NM522/pNT2/pNT32) and *C. ammoniagenes* DN510 forming UTP from orotic acid. The reaction mixture contained *E. coli* NM522/pGT8 cells expressing the β1-4 galactosyltransferase gene of *Neisseria gonorrhoeae*, *E. coli* NM522/pNT2/pNT32 and *C. ammoniagenes* DN510 cells. Orotic

acid, GlcNAc, as well as galactose, were added to the reaction mixture. It was shown that GlcNAc is transported to *E. coli* strains by the action of nag genes.[82-83] After 38 hours, these cells produced 107 g l^{-1} (279 mM) of LacNAc from 578 mM galactose and 290 mM GlcNAc, giving a molar yield of 48% based on galactose and 96% based on GlcNAc. Despite these high yields, the long reaction times (38 hours) led to a lower productivity and may potentially restrict commercialization.

A metabolically engineered *E. coli* strain (*E. coli* JM109) lacking the β-galactosidase gene (*lacZ*) was also used for the effective synthesis of lacto-*N*-triose II (LNT2).[78] As shown in Figure 4(B), the β-galactoside permease LacY transported lactose into the cell but due to the removal of hydrolytic activity (deletion of the *lacZ* gene), lactose was not converted to glucose and galactose. Under these conditions LNT2 (6 g l^{-1}) was obtained, due to the expression of a *lgtA* gene of *Neisseria meningitidis* encoding a β1-3 *N*-acetylglucosaminyltransferase with the use of an endogenous UDP-*N*-acetylglucosamine. The cultivation of these engineered bacteria at high densities was performed using glycerol as the carbon and energy source. LNnT was produced, together with other poly-LacNAc structures, including lacto-*N*-neohexaose (LNnH), lacto-*N*-neooctaose (LNnO) and lacto-*N*-neodecaose (LNnD) due to the co-expression of the *lgtB* gene encoding a β1-4 galactosyltransferase with the *lgtA* gene in the JM109 strain. In this case, the strain JM109 was grown on a glucose medium instead of glycerol owing to the toxicity resulting from the overexpression of the two genes on a glycerol medium. The target oligosaccharides accumulated inside the cells and were not released to the extracellular medium due to their large sizes. Therefore, these intracellular products were purified by charcoal adsorption during subsequent purification of the LNT2 product.

(Figure 4)

In a study by Bettler et al.[84] the Azorhizobium chitin pentaose synthase NodC (a β1-4 GlcNAc-transferase) and the *lgtB* β1-4 galactosyltransferase (a lactosamine synthase) from *Neisseria meningitidis* were also co-expressed in *E. coli*. The recombinant strain was cultivated for the biosynthesis of *N*-acetyllactosamine-containing oligosaccharides. The total concentration of oligosaccharides was 1.35 g l^{-1} after 40 hours of cultivation.

No wild type microorganism has been reported to generate LacNAc-derived structures naturally, indicating that the need for genetic modification is inevitable[85] Microbial production of more complex oligosaccharides necessitates the expression of further genes[84] and optimization of these systems for high productivity. The disadvantage of this approach, employing whole cells, is that these systems can be difficult to control and optimize, with undesirable side reactions, product or substrate inhibitory effects, limitations in the uptake of high concentrations of substrates and complex culture media, requiring research to optimize these systems. In addition, large fermenters (over 200 m^3) generate gradients of carbon source, oxygen and pH, making the control of carbon source feeding rate, as well as prevention of overflow or anerobic metabolism more challenging. In addition, the formation of inhibitory metabolites and environmental stresses can lead to lower biomass yields.[5] Whole cell systems are therefore typically employed when numerous enzymes in multi-step reactions are required and an engineered host cell can offer advantages or when enzyme production and isolation is difficult and costly.[5, 86-88]

In vitro enzymatic synthesis

Enzymes obtained from bacteria, fungi and yeasts are commonly utilized to synthesize LacNAc and related compounds.[89-90] These enzymes, which are responsible for forming a new glycosidic bond in oligosaccharides, are categorized into two groups: glycoside hydrolases with

transglycosylation ability and glycosyltransferases; which are different in terms of production source, yield, mechanism, regio-selectivity and substrate specificity. Excellent reviews are available that focus on both glycoside hydrolases and glycosyltransferases.[91-95] The transferases are more stereo-selective but have until recently been considered difficult to express recombinantly and require expensive starting substrates, notably nucleotide sugars. Glycoside hydrolases, on the other hand, can act on cheap substrates. Glycoside hydrolases are also more stable and accessible; however, they drive a hydrolytic reaction pathway in addition to a synthetic pathway and they have often lower synthetic yields compared to glycosyltransferases.

Glycosyltransferases for synthesis of LacNAc-based molecules

A great deal of research has focused on the identification, purification and expression of glycosyltransferase enzymes due to their extensive biological functions, especially in glycosylation of proteins and carbohydrates. To date, over 300 glycosyltransferases from mammalian tissues and more than 110 glycogens, purified and cloned from disparate *in vivo* sources such as rats, humans, mice and cattle, have been identified and analyzed.[96-97] In addition, such enzymes have been successfully produced in and purified from a wide variety of microbial sources.[3, 97-98]

Based on the type of linkage formed and substrate specificity, different families of galactosyltransferases have been identified. β1-3 galactosyltransferase (β3GalT; GT31) genes are responsible for the expression of enzymes transferring Gal unit to the β1-3 position of GlcNAc,[99] with the Galβ1→4GlcNAc (LacNAc) moiety being prevalent in many oligosaccharides. β1-4 galactosyltransferase (β4GalT; GT7) enzymes catalyze the transfer of galactose from UDP-Gal to GlcNAc.[100] The transfer of GlcNAc from UDP-GlcNAc to Gal is also directed by β1-3 *N*-

acetylglucosaminyltransferase (iGnT; GT14). Various core LacNAc-containing oligosaccharides can also be synthesized by β4GalT and iGnT starting from lactose (Figure 5).

(Figure 5)

Glycosyltransferases from mammalian sources differ significantly from those from bacterial sources in their expression system, structure, solubility, stability and substrate specificity. A comprehensive comparison between such mammalian and bacterial glycosyltransferases is provided in reviews by Bode et al.[101] and Brockhausen.[102] The isolation of mammalian glycosyltransferases as integral membrane proteins, most of which are in the Golgi apparatus, is difficult due to the need for solubilization with detergents and stability issues. On the other hand, some bacterial glycosyltransferases are soluble proteins with no membrane association, facilitating the isolation process and making successful recombinant expression easier. The expression of mammalian glycosyltransferases in bacterial hosts is also challenging, because of the lack of organelle membrane structures in such hosts, the absence of post translational modifications and differences in the oxidative environment during folding processes. In contrast, bacterial glycosyltransferases can now often be expressed in relevant *E. coli* hosts.[101-102] With this in mind, recombinant bacterial glycosyltransferases have been developed as potential candidates for LacNAc-related synthesis in many studies.

The product yield can be high in reactions catalyzed by such glycosyltransferases. For example, a mixture of enzymes including a galactosyltransferase (EC 2.4.1.22) and accompanying enzymes, normally known as ground working enzymes, (UDP-galactose 4'-epimerase (EC 5.1.3.2), UDP-glucose pyrophosphorylase (EC 23.7.9), phosphoglucomutase (EC 2.7.5.1) and pyruvate kinase (EC 2.7.1.40)) using different substrates, including glucose 6-phosphate (50 mM),

UDP (0.625 mM), GlcNAc (50 mM) and phosphoenolpyruvate (52.5 mM), resulted in a molar yield of 85% LacNAc based on glucose 6-phosphate after 96 hours at room temperature, followed by treatment with ion exchange resins.[103] The isolation yield after further purification by gel permeation chromatography was 70%. In another example, Yu et al.[104] developed a sequential one-pot multi-enzyme synthesis of LNT2 (isolation yield of 95%) and LNnT (isolation yield of 92%) from lactose. The products obtained were further elongated to two sialylated HMOs, α2-6-linked disialyllacto-*N*-neotetraose (DSLNnT) (isolation yield 99%) and α2-6-linked disialyllacto-*N*-tetraose (DS'LNT) with an isolation yield of 98%. This multi-enzyme system included several types of glycosyltransferases, namely *N*-acetylglucosamineuridyltransferase from *Pasteurella multocida*, β1-3 *N*-acetylglucosaminyltransferase from *Neisseria meningitidis*, β1-4 galactosyltransferase from *Neisseria meningitidis* and α2-6 sialyltransferase from *Photobacterium damselae*. Chen et al.[12] developed an enzymatic modular assembly with the aid of bacterial glycosyltransferases, including β1-3 *N*-acetylglucosaminylation (*Helicobacter pylori*), β1-4 galactosylation (*Neisseria meningitidis*), α1,3 fucosylation (*Helicobacter pylori*) and α2-3 sialylation (*Pasteurella multocida*), for the synthesis of thirty three poly-LacNAc derivatives starting from LNnT. In most cases, the oligosaccharide yields were more than 90%, illustrating the potential of this approach.

The key issue and the potential barrier for large-scale production using glycosyltransferases is the requirement for regeneration of the sugar nucleotide glycosyl donor. One potential solution to this barrier is the production of LacNAc with *in situ* regeneration of uridine-5'-diphosphate glucose (UDP-Glc) and uridine-5'-diphosphate galactose (UDP-Gal).[105] A system of three enzymes, sucrose synthase (from rice grains), UDP-Glc 4'-epimerase (from *Saccharomyces cerevisiae)* and β1-4 galactosyltransferase (from human milk), was used with a complex mixture of different

substrates including sucrose (500 mM), UDP-Glc (10 mM), GlcNAc (100 mM) and dUDP or dTDP-6-deoxy-D-xylo-4-hexulose (1 mM) for the synthesis of around 350 mg LacNAc (Figure 6(A)). A multistep isolation method was then employed to separate the components in the reaction medium. This commenced with enzymatic cleavage of sucrose to glucose and fructose using invertase from yeast; and continued with separation of LacNAc from monosaccharides by ligand exchange chromatography with a cation exchanger in Ca^{2+}-form; anion-exchange chromatography for the removal of nucleotides, nucleotide sugars, as well as buffer salt; and was completed with gel filtration for the removal of residual glucose and GlcNAc. To reduce the cost of the enzymes and improve productivity, ultrafiltration was used to separate the product from the enzyme, which was retained in the reaction medium (Figure 6(B)). Fresh substrate was then added and the method repeated over 11 days in 11 batches. While the LacNAc yield obtained after 22 hours was almost 100% in the first batch, it decreased to 40-50% with a longer incubation time (30 hours) after 11 batches due to inactivation of the enzyme during the synthesis and filtration. The average LacNAc yield was 57.4%. Transition-state analogs dUDP- or dTDP-6-deoxy-D-xylo-4-hexulose were also added to the mixture to reactivate the UDP-glucose 4'-epimerase.

(Figure 6)

In another substrate regeneration study by Chen et al.[106] a system of multiple immobilized enzymes was created for UDP-Gal regeneration. Four enzymes: galactokinase (GalK; EC 2.7.1.6), galactose-1-phosphate uridylyltransferase (GalPUT; EC 2.7.7.12), glucose-1-phosphate uridylyltransferase (GalU; EC 2.7.7.9) and pyruvate kinase (PykF; EC 2.7.1.40) were immobilized onto Ni^{2+}-NTA agarose beads. The beads were then incubated with a bovine β1-4 galactosyltransferase and a mixture of GlcNAc (0.24 mmol), ATP (24 µmol), phosphoenolpyruvate (0.48 mmol), UDP (24 µmol), glucose-1-phosphate (24 µmol) and galactose

(0.3 mmol). After reaction, the beads were separated and the LacNAc product was purified using anion exchange followed by gel permeation chromatography. The total amount of LacNAc obtained was 85 mg (0.22 mmol) giving a yield of 92%. This regeneration system enables the reuse of the enzymes but the long reaction time (four days) might hamper large-scale synthesis.

Tasi et al.[107] developed different sugar nucleotide regeneration systems using multiple enzymes including GalK, UDP-sugar pyrophosphorylase (AtUSP), pyruvate kinase (PK) and inorganic pyrophosphatase (PPA) for production of a UDP-Gal regeneration system; *N*-acetylhexosamine kinase (NahK), *N*-acetyl glucosamine-1-phosphate uridyltransferase (GlmU), PK and PPA for production of a UDP-*N*-acetylgalactosamine (UDP-GalNAc) regeneration system; bifunctional fucokinase/GDP-L-fucose pyrophosphorylase (FKP), PK and PPA for production of a GDP-L-fucose (GDP-Fuc) regeneration system; and cytidine monophosphate kinase (CMK), CMP-sialic acid synthetase (CSS), PK and PPA for production of a cytidine-5'-monophosphate-5-*N*-acetylneuraminic acid (CMP-Neu5Ac) regeneration system. These systems were successfully combined with other glycosyltransferases for oligosaccharide synthesis (not LacNAc based) at a gram scale. The long reaction time (between 3 to 10 days), however, could be problematic for a practical scale operation.

Sialyltransferases transfer sialic acid (*N*-acetylneuraminic acid) from the donor substrate CMP-Neu5Ac to various acceptors.[108-109] As shown in Figure 7, LacNAc can be used as an acceptor in a sialyltranferase-catalyzed reaction to form sialyl LacNAc with a yield of 89%.[110] This process includes *in situ* generation of sialic acid from *N*-acetylmannosamine (ManNAc) and pyruvate catalyzed by a sialic acid aldolase and coupled with *in situ* regeneration of the donor CMP-Neu5Ac catalyzed by a CMP-Neu5Ac synthase. To run the reaction, specific amounts of ManNAc, LacNAc, CMP, ATP, pyruvic acid, as well as enzymes including pyruvate kinase, nucleoside

monophosphate kinase, pyrophosphorylase, sialyl aldolase, CMP-Neu5Ac synthase and α2-6 sialyltransferase were added to the reaction buffer (HEPES; 200 mM, pH 7.5). The reaction was performed at room temperature for two days.

(Figure 7)

The exploitation of glycosyltransferases as tools for the synthesis of complex LacNAc-based extensions has been studied by several leading research groups. Paulson and co-workers.[111] reported the enzymatic synthesis of multi-sialylated poly-LacNAc glycans using a recombinant α2-6 sialyltransferase from *Photobacterium damselae* (Pd2,6ST) and CMP-Neu5Ac as the donor substrate. They synthesized different oligo- and poly-LacNAc acceptors based on previously developed glycosyltransferase-based methods.[112-113] These methods are of significant importance, particularly for the production of such poly-LacNAc extensions in quantities needed for detailed biological studies and the generation of glycan libraries. Nonetheless, further research is still needed to commercialize the advanced core LacNAc structures needed for elongation of poly-LacNAc chains in glycosyltransferase-catalyzed cascade reactions.

In a similar study by Lin and co-workers[114] a sequential enzymatic process was developed to synthesize oligo-LacNAcs followed by sialylation of oligo-LacNAcs at a milligram scale. They first synthesized LacNAc (46 mg) using a 6-azidohexyl-GlcNAc (azido-GlcNAc) acceptor and a UDP-Gal donor in a reaction catalyzed by a recombinant β1-4 galactosyltransferase from *Neisseria meningitidis*. The one-pot synthesis of UDP-Gal from galactose-1-phosphate (Gal-1-P) was performed by a thermophilic thymidylyltransferase (RmlA) from *Aneurinibacillus thermoaerophilus* coupled with a recombinant galactokinase (MtGalK) from *Meiothermus taiwanensis*. A β1,3-*N*-acetyl-glucosaminyltransferase from *Helicobacter pylori* (HpGnT) was

399 then used to transfer a GlcNAc moiety from UDP-GlcNAc to LacNAc to form a GlcNAc-LacNAc
400 trisaccharide. A regeneration system containing RmlA and *N*-acetylhexosamine kinase (NahK)
401 from *Bifidobacterium longum* was also employed to convert GlcNAc to UDP-GlcNAc. Other
402 LacNAc oligomers of defined length were synthesized in a manner similar to that described for
403 LacNAc and the GlcNAc-LacNAc trisaccharide. The oligo-LacNAcs obtained were then sialylated
404 using CMP-Neu5Ac catalyzed by an α2-6 sialyltransferase (Pd2,6ST from *Photobacterium*
405 *damselae*) or an α2-3 sialyltransferase (PmST from *Pasteurella multocida*).

406 Yu and co-workers[7] have recently used a sequential one-pot glycosyltransferase-catalyzed
407 synthesis coupled with the same UDP-Gal and UDP-GlcNAc regeneration systems developed by
408 Lin and co-workers[114] to produce core LacNAc-containing oligosaccharides, including *p*-LNH
409 (see Figure 5), at milligram scale. In this method, lacto-*N*-triose (165 mg) was first synthesized via
410 an enzymatic reaction between lactose and UDP-GlcNAc catalyzed by a β1,3 *N*-
411 acetylglucosaminyltransferase from *H. pylori* strain 26695 (HP1105). A β1,4 galactosyltransferase
412 from *H. pylori* strain NCTC11637 (HP0826) was used to galactosylate lacto-*N*-triose with UDP-
413 Gal to produce LNnT (58 mg). The LNnT was converted to a pentasaccharide product using
414 HP1105 in the presence of UDP-GlcNAc. The treatment of the resulting pentasaccharide with
415 UDP-Gal catalyzed by HP0826 produced *p*-LNnH (59 mg) after 93 hours. In addition, a β1,3
416 galactosyltransferase from *E. coli* O55:H7 converted the pentasaccharide to *p*-LNH (45 mg) in the
417 presence of UDP-Gal after 14 hours.

418 With broad substrate tolerance for LacNAc containing glycans, bacterial fucosyltransferases
419 are versatile enzymes for the synthesis of (multi)fucosylated glycans.[115-117] Following the work by
420 Yu and co-workers[7] described above, the KH-1 antigen has recently been produced by the same
421 group[115] via multi-fucosylation of *p*-LNnH in a sequential enzymatic reaction. They reacted 20 mg

of *p*-LNnH with GDP-Fuc by an α1,2 fucosyltransferase from *H. pylori* (HpFutC) for 3 hours to obtain branched fucosyl *para*-lacto-*N*-hexaose (F-*p*LNnH). After the reaction, a fucosyltransferase from *Bacteroides fragilis* NCTC 9343 (Bf13FT) was added to the solution containing F-*p*LNnH. After 6 hours, a mixture of fucosylated products including two inseparable difucosylated isomers (0.5 mg), the KH-1 antigen (16.2 mg) and one tetra-fucosylated product (6.6 mg) was obtained. Although the overall sequential reaction time was 103 hours, this study demonstrates the potential importance of bacterial glycosyltrasferases for the synthesis of such complex LacNAc-based structures.

Huang et al.[73] have recently synthesized imidazolium-tagged LacNAc (LacNAc-ITag) and *p*NP-β-LacNAc (Figure 8) from lactose in the presence of UDP using a recombinant β1-4 galactosyltransferase (β4GalT) from *Neisseria meningitidis* serogroup B strain MC58 (NmLgtB-B). Instead of adding UDP-Gal externally (Pathway 1 in Figure 8(A)), UDP-Gal was directly generated from lactose and UDP without the utilization of any additional cofactor (Pathway 2 in Figure 8(A)). GlcNAc-ITag was chemically synthesized from 1-azidopropyl derivatives in four steps. The use of imidazolium-based probes (I-Tags) also allowed the analysis of glycosylation reactions, even in these complex mixtures, due to their greater spectral peak intensities and lower limits of detection by mass spectrometry. The LacNAc-ITag produced could also further be used for the synthesis of imidazolium-tagged sialyl-LacNAc probes using sialyltransferases.[118] Furthermore, the *p*NP-β-LacNAc produced is a starting substrate for the synthesis of other complex molecules.[119-121]

(Figure 8)

Although glycosyltransferase-catalyzed reactions result in a target product with high selectivity and high yield, these enzymes are not readily available in naturally occurring microbial systems and so must commonly be obtained from genetically modified microorganisms, making them expensive. Further, these enzyme systems need a nucleotide donor, which is unstable and also expensive. Nonetheless, for the enzymatic synthesis of more complex LacNAc extensions, glycosyltransferases, in particular those derived from bacterial sources with wide substrate tolerance, play a major role. Thus, a commercial source of LacNAc, core LacNAc-based molecules as well as nucleotide donors could facilitate the synthesis of more complex oligosaccharides in sequential glycosyltransferase catalyzed reactions from a discovery to a large scale. Regenerative strategies and synthesis routes for nucleotide sugars have already been discussed well in several reviews.[122-124] Further studies are still needed to validate the scalability and economic feasibility of these routes which are influenced by the number of enzymes required, expression as well as purification complexity, the number of regeneration cycles, cofactor requirements and the choice of starting substrates.[122]

In summary, the *in situ* regeneration method for LacNAc production is complex, as shown in Figure 6. Further, the purification methods needed following production restrict large-scale application. An alternative approach is to carry out transgalactosylation reactions catalyzed by β-galactosidases, which is discussed in the following sections.

Glycoside hydrolases for synthesis of LacNAc-based molecules

β-Galactosidases

β-Galactosidases are categorized into glycoside hydrolase (GH) families. β-Galactosidases from glycoside hydrolyse families of 1, 2, 3, 35, 42, 50 and 59 have a triosephosphate isomerase (TIM)-barrel structure for the catalytic domain with two glutamic acid residues acting as an acid/base catalyst.[125] Among these are the commercially important β-galactosidases from *Bacillus circulans* (GH2 family), *Aspergillus oryzae* (GH35 family), *Kluyveromyces lactis* (GH2 family), *Kluyveromyces fragilis* (GH2 family) and *Streptococcus thermophilus* (GH2 family), which have been used for the synthesis of galactooligosaccharides (GOS).[126] The largest number of β-galactosidases are classified into the GH2 family and the enzymes in this category show increasingly high hydrolysis and transgalactosylation activities as more are discovered.[127] The GH family 2 β-galactosidase from *Bacillus circulans* (BgaD), specifically shows high transglycosylation activity for the synthesis of lactose-derived compounds. This enzyme can effectively transfer galactosyl from a donor substrate onto various acceptors.[128]

A potential disadvantage of these enzymes is the number of reaction pathways that can be driven by the β-galactosidase enzyme, reducing selectivity and the yield of the target prebiotic (Figure 9(A)). In particular, transgalactosylation usually competes with hydrolysis. During lactose hydrolysis, glucose is released, leaving a galactosyl moiety that interacts with the enzyme active site. This moiety is then linked to an acceptor containing a hydroxyl group. A hydrolysis reaction occurs when water molecules act as the acceptor. Nevertheless, if the acceptor is another sugar molecule, a transgalactosylation reaction is instigated. The experimentally determined outputs from the β-galactosidase-catalyzed manufacture of LacNAc from lactose or nitrophenyl donors are

485 shown in Table 1. As can be seen, molar yields based on the donor substate depend upon the type
486 of enzyme, donor and acceptor concentrations, as well as the ratio of these molecules, with yields
487 ranging from 0 to 66%. Nevertheless, nitrophenyl glycosides are a more costly substrate than
488 lactose and not suitable for food applications, given their toxicity.[129]

489 **(Figure 9)**

490

Table 1. Synthesis of LacNAc from lactose or phenyl-D-galactopyranosides as donors and GlcNAc as acceptor, using β-galactosidase enzymes.

Enzyme source	Donor	Donor concentration (mM)	GlcNAc acceptor concentration (mM)	Reaction time (h)	Temperature (°C)	Molar yield based on lactose[i] (%)	Molar yield based on NPG[i] (%)	LacNAc produced[ii] (mg)	Reference
B. circulans	Lactose	50	99	24	30	25 (isolation yield)		5100	130
B. circulans	Lactose	500	500	4	40	29		55	131
B. circulans	Lactose	730	230	3	15	13 (isolation yield)		40	132
B. circulans	Lactose	1000	250	1	40	4		na	133
B. circulans	oNPG	50	300	0.5	25		25	14	134
B. circulans	oNPG	33	433	1	55		46	6	135
B. circulans	oNPG	50	1000	1	40		65.5	na	136
B. circulans	pNPG	330	2480	1	30		30	38	128
B. circulans	pNPG	33	433	2	55		74	10	135
B. circulans	Phenyl-D-galactopyranoside	33	433	5	55		14	2	135
A. oryzae	Lactose	1000	1000		60	1.9		7	137
A. oryzae	oNPG	50	300	0.5	25		0	0	134
K. lactis	Lactose	1000	250	1	40	0.63		na	133
T. thermophilus (cell extracts)	pNPG	170	830		80		41	27	138
T. thermophilus (pure enzyme)	pNPG	170	830		65		34	22	138
T. thermophilus	pNPG	170	850	3	60		17	11	139
B. bifidum	Lactose	1500	4500	10	37	20 (isolation yield)		109	74
E. coli	oNPG	50	300	0.5	25		0	0	134
S. solfataricus	Lactose	1000	1000		75	5.2		20	137

[i]Yield based on HPLC analysis of crude product, unless specified as the yield after isolation.
[ii]The LacNAc produced was calculated based on the reaction volume where available. na: not available

Transglycosylation catalyzed by β-galactosidases is affected by several factors, including the substrate concentration and specificity, cation concentration, acceptor to donor ratio and temperature. These are considered here in turn.

Substrate concentration and specificity

It has been reported that the initial substrate concentration in enzymatic transglycosylation reactions can affect the product yield.[140-141] In addition, the initial substrate concentration may change the product composition.[142] Li et al.[131] showed that at lactose concentrations greater than 1 M, the yield of LacNAc decreased, while the production of Allo-LacNAc increased with the increase of substrate concentration. These effects may be enzyme specific, however, as it was also concluded that β-galactosidase from *L. bulgaricus* was not inhibited by its substrates, i.e. lactose at concentrations up to 600 mM or 2-nitrophenyl β-D-galactopyranoside (*o*NPG) at concentrations up to 25 mM.[143] Furthermore, many β-galactosidases show different affinities to various substrates. These enzymes are specific to the β-D-glycosidic linkage and display a better specificity for galactosides containing *ortho*- or *para*-nitrophenyl groups rather than methyl or phenyl groups.[144-145]

Cations

Whey is a source of lactose that can be used directly for performing an *in situ* transgalactosylation reaction. The existence of cations, namely sodium, potassium, magnesium and calcium in the whey, however, may affect the enzymatic reaction. Depending on the biocatalyst source, cations may affect the hydrolytic activity in different ways. For example, divalent cations, including Mn^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+} and Ca^{2+}, enhanced the hydrolytic activity of a thermostable recombinant β-galactosidase from *Thermotoga maritima* measured at its optimum

520 temperature (80 °C) for 5 min,[146] while the activity of a recombinant β-galactosidase from
521 *Bifidobacterium infantis* HL96 measured at 60 °C for 10 min was inhibited by these cations.[147]

522 While the references above measured the hydrolytic activity of β-galactosidases using the
523 colorimetric substrate *o*NPG, both hydrolysis and transgalactosylation occur simultaneously when
524 glycoside hydrolases are used. The cation concentration can affect both these simultaneous
525 reactions to a differing extent, leading to changes in product composition. In a study by Fischer
526 and Kleinschmidt[148] the activity of *Kluyveromyces lactis* β-galactosidase significantly increased
527 (up to 90 fold) even at low concentrations of the cations (1 mM-10 mM) commonly found in whey,
528 i.e. K^+, Na^+, Mg^{2+}. Nonetheless, the GOS yield in whey decreased and a different product
529 composition was obtained compared to a lactose solution with the same initial lactose
530 concentration. Huber et al.[149] showed that Ca^{2+} did not activate β-galactosidase from *Escherichia*
531 *coli*. They demonstrated, however, that Ca^{2+} did bind to this enzyme and the transgalactosylation
532 and hydrolysis rates varied when it was present. In our own recent work, the activity of *Bacillus*
533 *circulans* β-galactosidase measured at 40 °C for 4 min was not significantly altered in the presence
534 of K^+, Na^+, Mg^{2+} and Ca^{2+}, under transgalactosylation reaction conditions (at 50 °C up to 120 min).
535 When divalent cations Mg^{2+} and Ca^{2+} were added at concentrations of 100 mM, however, there
536 was a significant reduction in the β-galactosidase activity, with a concurrent change in product
537 selectivity and yield. These changes were attributed to the formation of enzyme aggregates, which
538 further influenced the hydrolysis and transgalactosylation pathways in the conversion of lactose to
539 LacNAc using this β-galactosidase.[150]

540

Water activity

When water activity decreases, the hydrolysis of substrates or secondary hydrolysis of products is less favored, thereby increasing the selectivity of transgalactosylation. Bridiau et al.[136] examined the effect of different hydro-organic solvents for the synthesis of LacNAc starting from *o*NPG. The maximum stability of β-galactosidase was obtained when 10% v/v tert-butyl alcohol was employed. They also found that the optimal yield was obtained when water activity was reduced to 0.96, irrespective of the organic solvent used; compared to pure water in which very few transgalactosylation products were formed. For water activities less than 0.9, the synthesis yield and the regioselectivity of the enzyme deteriorated due to an increase in the enzyme structural rigidity. Sandoval et al.[138] showed that the enzyme became more efficient in an ionic liquid–water mixture. They achieved a LacNAc yield of 34% in a buffer solution, while the yield increased to 79% when 30% v/v 1-octyl-3-methylimidazolium hexafluorophosphate ([Omim][PF6]) in buffer was used. Similarly, the use of bio-solvents including glycerol and 3-*N*,*N*-dimethyl amide based solvents, at an optimal concentration of 2 M, increased LacNAc production and restricted undesired side reactions.[139] It was concluded that conformational changes in the secondary and tertiary structures of the enzyme were responsible for improving the enzymatic reactions. While the use of such co-solvents can increase yield, they will also add substantially to raw material costs, their toxicity may limit use in food systems and they may require significantly more complex manufacturing infrastructure due to safety concerns regarding flammability.

Temperature and thermostable enzymes

562 Working at higher temperatures can lead to improved transgalactosylation. Zeuner et al.[8]
563 expressed three types of thermostable β-galactosidases from *Bacillus circulans* (BgaD-D),
564 *Thermus thermophilus* HB27 (Ttβ-gly) and *Pyrococcus furiosus* (CelB) for the production of
565 LNnT from lactose and LNT2, as well as LacNAc from lactose and *N*-acetylglucosamine. The
566 optimum temperature and time were at 90 °C and 10 min for CelB, 65 °C and 30 min for Ttβ-gly
567 and 50 °C and 30 min for BgaD-D. Despite being less stable at higher temperatures, BgaD-D was
568 more efficient in terms of the product yield, producing two-fold and six-fold more product than
569 Ttβ-gly and CelB, respectively. Working at an elevated temperature, however, improved the yield
570 of LacNAc by 5 times (BgaD-D) and 6 times (Ttβ-gly and CelB) relative to the yield at 40 °C.

571 The use of hyperthermophiles—*Sulfolobus solfataricus* (LacS) and *Pyrococcus furiosus*
572 (CelB)— in transgalactosylation reactions was also investigated by Hansson and Adlercreutz[151]
573 They worked at temperatures up to 95 °C, at which lactose concentrations could be increased up
574 to 90% (w/v). The maximum yield continuously increased as temperature increased to 85 °C for
575 LacS and 95 °C for CelB. Nonetheless, very high lactose concentrations and temperatures did not
576 always enhance transgalactosylation. The synthesis at temperatures higher than 75 °C and
577 concentrations greater than 70% lactose led to enzyme inactivation due to Maillard reactions, in
578 which brown-colored compounds were formed as a result of reactions between the protein amino
579 residues and reducing sugars.

580

Sialidases

Sialic acids are another interesting functionality that may be transferred onto LacNAc-based structures. The biological functions of numerous sialo-glycoconjugates on the human cell surface are controlled by sialic acids, including *N*-acetylneuraminic acid (Neu5Ac), deaminated neuraminic acid (KDN), *N*-glycolylneuraminic acid (Neu5Gc) and neuraminic acid (Neu), with around 50 sialic acid derivatives discovered to date. The majority of these are Neu5Ac in an α2-3- or α2-6-linkage to galactose (Gal) (Figure 9(B-D)).[80, 152] There are also other dominant structures including an α2-3- or α2-6-linkage to *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GlcNAc) or an α2-8-linkage to another *N*-acetylneuraminic acid at the terminal position of glycans.[153]

Sialidases catalyzing the transfer of a sialyl moiety onto an acceptor such as LacNAc are regarded as trans-sialidases. Sialyltransferases require an expensive donor CMP-Neu5Ac, and there are additional complexities associated with the *in situ* regeneration reactions catalyzed by these transferase enzymes (Figure 7). In contrast, trans-sialidases can act on various donors. Similar to β-galactosidases, the reaction mechanism consists of both trans-sialylation and hydrolysis (Figure 9(E)). *p*NP-α-sialic acid (Sia-α-*p*NP), 3′-sialyl-lactose, 2′-(4-methylumbelliferyl)-α-D-*N*-acetyl neuraminic acid (Sia-α-MU) and casein glycomacropeptide (CGMP) are typically used as donors, providing sialic acid (*N*-acetylneuraminic acid).[154-155] Because CGMP can be commercially derived from dairy products and by-products, including whey, it is a cheaper sialyl donor (5-11% of CGMP is sialic acid) and more attractive for large-scale production[129, 156] than the alternative donor sources.

Sialidases with disparate kinetics, substrate specificity and regioselectivity can be obtained from various bacterial strains, including *Arthrobacter nicotianae, Arthrobacter ureafaciens, Clostridium perfringens, Pasteurella multocida and Streptococcus pneumoniae*.[157] Displaying a high trans-sialylation activity, sialidases from *Trypanosoma* species, such as *Trypanosoma cruzi*, *Trypanosoma congolense* and *Trypanosoma brucei*, have substrate specificities to different acceptors. The sialidase from *T. cruzi* can only act on substrates with the α2-3-linked sialyl residues,[158] while the sialidase from *Arthrobacter sialophilus* is specific for either of the α2-3-, α2-6- or α2-8-linked sialic acids.[157] Sialidases from *Clostridium perfringens, Arthrobacter ureafaciens, Vibrio cholerae* and *Newcastle disease virus* can also hydrolyze α2-8-linked sialic acids.[129] Compared to sialyltransferases, the yield of sialylation product is low (generally <30%) for most sialidases derived from different sources. The trans-sialidase from *T. cruzi*, however, has been shown to have much higher yields (up to 80%) depending on the donor substrate.[129, 159-160]

Scudder et al.**[161]** reported that *T. cruzi* trans-sialidase is able to sialylate a wide range of substrates with subterminal β1-4Man, β1-4Ga1, βl-6Gal and β1-6GlcNAc as well as LacNAc, LNnT, LNT and lacto-*N*-fucopentaose V (LNFP V). They showed that starting with LNT as the acceptor substrate and sialyllactose as the sialyl donor, *T. cruzi* trans-sialidase converted 66% of LNT to a precursor of sialyl-Lewis^{x} and sialyl-Lewis^{a}. Michalak et al.**[162]** successfully modified a sialidase derived from *Trypanosoma rangeli* with superior trans-sialidase activity. They expressed the enzyme in *Pichia pastoris* and then used it for sialylation of LNT, LNnT, lacto-*N*-fucopentaose I (LNFP I) and LNFPV. A total of 3.6 g 3′-sialyllactose was synthesized in a pilot scale (5 l) reactor using CGMP as the sialyl donor and lactose as the acceptor after 20 min. Trans-sialylation of LNT, LNnT, LNFP I and LNFPV as the acceptors and CGMP as the donor was performed in a 5-100 ml scale reactor which resulted in 5-20 mg of sialylated oligosaccharides. These examples are

indicative of the potential of trans-sialidases for the production of various sialylated saccharides, as well as sialylated LacNAc structures. Protein engineering will also likely play a role in improving the trans-sialylation activity of these enzymes and facilitating the scale-up.

β-*N*-Acetylhexosaminidases

Some extended LacNAc-core structures can be enzymatically synthesized by the action of a group of glycoside hydrolases known as β-*N*-acetylhexosaminidases. β-*N*-Acetylhexosaminidases are GH20 glycoside hydrolases that catalyse cleavage of terminal, non-reducing *N*-acetylhexosamine moieties and can catalyze the hydrolysis of *N*-acetylglucosides or *N*-acetylgalactosides containing a 2-acetamido group.[163-164] They have attracted attention due to their inherent ability to catalyze transglycosylation reactions in which *N*-acetylhexosamine residues, namely GlcNAc or GalNAc, are transferred from a donor substrate to an acceptor substrate, creating more complex bioactive carbohydrates.[165-169] Artificial *para*-nitrophenyl donors including *para*-nitrophenyl *N*-acetyl-β-D-glucosaminide (*p*NP-β-GlcNAc) and *para*-nitrophenyl *N*-acetyl-β-D-galactosaminide (*p*NP-β-GalNAc) have been used widely in β-*N*-acetylhexosaminidase-catalyzed transglycosylation reactions.[170-173] In addition, a wide variety of other synthetic donors such as 4-deoxy-substrates,[174] 1,2-oxazoline containing substrates[169, 175] and glycosyl azides[176] have been developed by researchers to improve such reactions via enhancing yields, substrate specificity and regioselectivity. Nonetheless, the groups released from artificial substrates after transferring GlcNAc or GalNAc moieties to an acceptor are often toxic and not suitable for food applications.[163] The use of *N,N'*-diacetylchitobiose ($(GlcNAc)_2$) has been investigated in some studies for transglycosylation of lactose catalyzed by β-*N*-acetylhexosaminidases,[8, 177-178] as it can be obtained as a naturally available, non-toxic and potentially cheaper substrate from chitin depolymerization.[179] In a study by Murata et al.[180] β-*N*-acetyl-D-hexosaminidase from *Nocardia*

orientalis was used to transfer *N*-acetylglucosaminyl from $(GlcNAc)_2$ to *p*NP-β-LacNAc to produce 3′-*O*- and 6′-*O*-*N*-acetylglucosaminyl-*N*-acetyllactosaminide glycosides (GlcNAc-LacNAc-*p*NP), which are believed to carry blood group ABH, Lewis and Ii antigens and act as structural units of polylactosamino glycans. The overall yield and the enzyme regioselectivity were varied by the addition of α-cyclodextrin. This overall yield was relatively low, however, varying between 2.9% to 7.4% using the acceptor *p*NP-β-LacNAc.

Certain β-*N*-acetylhexosaminidases can catalyse synthesis of lacto-*N*-triose II (LNT2) from lactose via reaction with a suitable *N*-acetylglucosamine source. LNT2 is an important HMO and a precursor for the synthesis of lacto-*N*-neotetraose (LNnT) via a β-galactosidase-catalyzed transgalactosylation reaction of lactose (Figure 10). LNnT is one of the key *N*-acetyllactosamine-containing carbohydrates found in human milk in large quantities and is approved for addition to infant and young child food products.[181-183] This is an interesting strategy to exploit lactose as a cheap and abundant substrate in successive transglycosylation reactions driven by two glycoside hydrolases. Depending on the type of enzyme and donor, various LNT2 yields from 2% to more than 80% have been reported.[163] Schmölzer et al.[169] have recently produced an engineered exo-hexosaminidase from *Bifidobacterium bifidum* incapable of hydrolyzing the LNnT formed, with a yield of 90% in less than 1 hour. They chemically synthesized *N*-acetyl-D-glucosamine 1,2-oxazoline (GlcNAc oxaline) from GlcNAc and then used this molecule as a donor for the production of LNT2 via β1-3 glycosylation of lactose.

(Figure 10)

ENZYME IMMOBILIZATION

The immobilization of enzymes can enhance stability, particularly towards conditions of extreme pH and elevated temperature. The bonds formed between enzymes and the support or carrier potentially reduce the conformational flexibility of the enzyme, thereby increasing resistance to thermal denaturation.[184] Further, it has been demonstrated that higher temperatures can increase the yield of oligosaccharide production[185] and this is possible with a more thermally stable immobilized enzyme.

Shiyan et al.[186] immobilized a trans-sialidase from *Trypanosoma cruzi* on ConA-Sepharose for α2-3 sialylation of the lactosamine type *N*-glycans. This immobilized enzyme was stored at 4 °C in the presence of 0.02% NaN_3 for several months without loss of activity and was successfully used for sialylation of oligosaccharides for five cycles.

Silica is an attractive support for enzyme immobilization due to its non-toxicity, microbial resistance, ease of handling and robustness towards high flow rates in continuous processes.[187-188] Engineered enzymes from *Trypanosoma rangeli* have been immobilized on two silica supports: glyoxyl-functionalized silica and silica functionalized with (3-aminopropyl)triethoxysilane (APTES), which were used for sialidase-catalyzed trans-sialylation by Zeuner et al.[189] While trans-sialylation activity was reduced after immobilization, the glyoxyl-functionalized silica system enhanced productivity by 50% relative to the free enzyme, as long as the enzyme was recycled more than six times. By contrast, glutaraldehyde-APTES-functionalized silica supports reduced the productivity to less than half that of the free sialidase. The glutaraldehyde-APTES method appears to generate a weaker linkage between sialidase and the support compared to the glyoxyl method,[189] potentially explaining these differences. This example also highlights how it is vital to

691 select a support capable of making strong bonds with the enzyme, if it is to be reused in successive
692 reactive cycles.

693 We recently used a layer-by-layer encapsulation method to immobilize β-galactosidase
694 onto silica particles.[190] In this technique, a positively charged layer of polyallylamine
695 hydrochloride (PAH) was first coated on the negatively charged silica particles, followed by the
696 electrostatic adsorption of the *Bacillus circulans* β-galactosidase (negatively charged at pH of 6),
697 glutaraldehyde crosslinking, as well as the additional deposition of oppositely charged
698 polyelectrolytes (PAH and polystyrene sulfonate (PSS)). This immobilized enzyme was used in at
699 least eight successive cycles of LacNAc synthesis from lactose with no significant decrease in the
700 LacNAc yield. Molecular analysis showed that changes in the enzyme activity resulted from
701 different interactions during the immobilization process. Staring with the same initial
702 transgalactosylation activities of the immobilized and free enzymes, a higher LacNAc yield was
703 obtained with the immobilized biocatalyst, leading to potentially improved economy and
704 sustainability when employed in a large-scale process.

705 Eskandarloo and Abbaspourrad[191] investigated the covalent association of β-galactosidase,
706 extracted from *Aspergillus oryzae*, to 3-aminopropyl triethoxysilane (3-APTES)-modified glass
707 beads and used these beads to continuously produce oligosaccharides via transgalactosylation of
708 lactose in whey permeates. The immobilized enzyme system was effective and only ~5% of the
709 oligosaccharide production yield was lost after eight reuses. In addition, the maximum temperature
710 for the optimal activity of the immobilized enzyme (60 °C) was greater than that for the free
711 enzyme (50 °C). About 51 % of the maximum immobilized enzyme activity was retained at 70 °C,
712 which is considerably higher observed with the free enzyme (10%). In spite of a 55% reduction in

713 the free β-galactosidase activity, the immobilized enzyme activity was around 89% of the initial
714 value after 240 min of incubation at 50 °C, illustrating the success of this approach.

715 The immobilization of glycosyltransferases has also been explored.[192-195] For example,
716 Schneider et al.[192] covalently immobilized a human milk β1-4 galactosyltransferase on both
717 CNBr-activated Sepharose 6MB and tresylchloride-activated Sepharose 4B. They also
718 investigated non-covalent attachment of this enzyme to Concanavalin A-Sepharose and
719 Protein G-Sepharose via affinity immobilization. This study indicates that more than 90% of
720 the initial activity of galactosyltransferase was lost after covalent immobilization, although
721 72% of the enzyme applied successfully formed a covalent bound with the supports. Around
722 50% of the initial soluble enzyme in the solution was non-covalently immobilized on the
723 affinity-based supports. The activity, however, still decreased significantly by 75%-79%
724 after this non-covalent immobilization. In contrast, the stability of the immobilized
725 galactosyltransferase improved compared to that of the free enzyme. After 65-hour storage
726 of the enzyme at room temperature in a buffer, the free enzyme lost 95% of its activity while
727 around 55% of the galactosyltransferase activity immobilized on either tresylchloride-
728 activated Sepharose 4B (covalent) or Concanavalin A-Sepharose (non-covalent) was
729 retained. In a study by Pišvejcová et al.[193] a β1,4 galactosyltransferase from bovine milk was
730 covalently immobilized on Eupergit C 250 L. They could successfully use the immobilized
731 galactosyltransferase in 13 cycles (24 h each) with about 40% of the initial activity retained at the
732 end of these reuse cycles.

733 Nevertheless, these studies have mainly focused on the immobilization of
734 galactosyltransferases. Further studies covering a wider range of glycosyltransferase

735 immobilization systems need to be undertaken, particularly those that have been developed for the
736 synthesis of LacNAc-based structures and relevant sugar nucleotide regeneration systems.

737 In a more recent report by Yu et al.[196] the site-specific covalent immobilization of a
738 sialyltransferase from *Pasteurella multocida* on PEGylated-N-terminal cysteine magnetic
739 nanoparticles (MNPs) was studied. They first synthesized three cysteine-functionalized MNPs by
740 modifying the surface of aminated Fe_3O_4 nanoparticles with different lengths of hydrophilic
741 ethylene glycol linkers. The activity measurement of the immobilized enzyme showed that 80%
742 of the sialyltransferase activity was retained after immobilization compared to the free counterpart.
743 More surprisingly, they found that the activity could be significantly improved by 225% when the
744 length of linker increased, mainly as a result of higher enzyme flexibility on the MNP surface with
745 an increased linker length. The immobilized enzyme was also reused in ten consecutive cycles
746 with 50% activity retention obtained after the final cycle. Given the lower robustness of
747 glycosyltransferases compared to glycoside hydrolases, these results are of significance, as they
748 demonstrate the improvement of sialyltransferases stability and activity upon the selection of an
749 appropriate immobilization technique.

750

751 ## PURIFICATION METHODS FOR LARGE-SCALE SYNTHESIS

752 Several purification techniques have been developed to separate the target LacNAc-based
753 molecule from other impurities at an industrial scale. Chromatographic separations using ion-
754 exchange absorbents can isolate non-prebiotic components, such as galactose, from probiotic
755 carbohydrates.[197-199] Adsorption onto activated charcoal has widely been used in the literature,
756 especially for large volumes.[200-201] The adsorbed carbohydrates can be eluted by different
757 concentrations of ethanol/methanol in water. For example, in the purification of LacNAc from a

758 solution containing other carbohydrates, GlcNAc can be first eluted with water, then lactose with
759 15% methanol, whilst LacNAc is eluted using methanol at a concentration of 20%.[202] Likewise, in
760 a study by Sakai et al.[130] LacNAc was first eluted with water and then with a linear gradient of 0-
761 30% ethanol, within a charcoal-Celite column. LNT2 and LNnT have also been purified by
762 adsorption onto activated charcoal followed by washing with water and elution with ethanol/water
763 solutions using a linear gradient, resulting in a purification yield of 60-70%.[78, 203] Activated
764 charcoal adsorption was also employed for the purification of the P1 trisaccharide[204] and
765 globotriose[205] at scale.

766 A selective crystallization step can be used after chromatographic purification to provide a
767 crystalline product.[206] A downstream processing method developed by the Glycom company (a
768 leading HMO supplier),[206-207] involves concentration by evaporation and/or nanofiltration after
769 chromatography, followed by the addition of a hot alcohol solvent, preferably methanol at 60 °C.
770 This mixture is then further concentrated and cooled, causing the *N*-acetyllactosamine-containing
771 oligosaccharide to precipitate as crystals. The use of methanol, however, can be problematic in
772 terms of waste treatment and storage costs due to the flammability of this solvent.[208]

773 As an alternative to expensive chromatographic purification methods, membrane
774 separation of galactooligosaccharides as a mixture from other low molecular weight sugars has
775 been investigated in numerous studies.[209-214] Given a typical molecular weight range of 200-1000
776 Da for commercial GOS mixtures, nanofiltration separation has predominantly been used in those
777 studies.[214] Likewise, nanofiltration can be applied to separate LacNAc-derived oligosaccharides,
778 which have molecular weights of more than 700 Da, from other low molecular weight
779 carbohydrates and other impurities. Chassagne et al.[215] developed a method based on membrane
780 filtration to separate neutral LacNAc-containing oligosaccharides, including LNnT, produced via

fermentation. In the first step, biomass and high molecular weight suspended solids were separated from soluble components by ultrafiltration. The UF permeate containing the neutral oligosaccharides was then subjected to nanofiltration to concentrate the oligosaccharides and reduce the content of inorganic salts coming from the fermentation broth. Additional treatments using ion exchange resins were also utilized to remove charged materials and color bodies.

The use of membrane technology for separation of a target carbohydrate rather than a mixture, however, is more challenging and less studied in the literature. Separation of sialylated oligosaccharides, such as sialyllactose, sialylated LacNAc and sialylated LNnT, from disaccharides and monosaccharides by nanofiltration has been investigated.[53, 216] Nordvang et al.[217] used a polyethersulfone nanofiltration membrane (NTR-7450) with a molecular weight cut-off of 600–800 Da and a zeta potential of −17 at pH=7 to efficiently separate sialyllactose (MW=633 Da) from lactose (MW=342 Da). They achieved a high retention of sialyllactose, close to 100%, as a result of both physical and electrostatic barriers. Both sialyllactose and the membrane were negatively charged at the working pH, which resulted in an electrostatic repulsion. Likewise, it is possible to separate sialyl LacNAc from LacNAc, as the molecular weight of sialyl LacNAc (675 Da) is greater than that of LacNAc (383 Da).

PERSPECTIVES

This review has highlighted the biological significance and wide application potential of LacNAc-based bioactives, showing that these bioactive molecules may be an important part of the future bioactive market and output of biotech companies. Despite extensive published research on the production of *N*-acetyllactosamine-containing carbohydrates, however, commercial manufacture faces many challenges. Direct extraction of LacNAc or its derivatives from milk is

803 complicated, as there are several molecules with similar structures to the target LacNAc or
804 LacNAc-derived molecule. Furthermore, the low concentrations of these compounds in milk adds
805 additional steps and costs to the downstream process. Chemical procedures for synthesis of
806 LacNAc-based compounds are complex and expensive owing to the many reaction steps involved,
807 the materials cost, limited availability of the specific reagents required and the wide range of by-
808 products produced during chemical reaction chains.

809 Among the techniques investigated in this review, biochemical routes are more
810 commercially attractive, as there is a possibility for the use of cheap and readily available
811 substrates. Microbial syntheses are suitable for the production of complex oligosaccharides but
812 enzymes are more efficient for the synthesis of core LacNAc-based structures, due to the simpler
813 reactions involved, with fewer by-products and impurities. There is also greater control of the
814 reaction pathways, particularly when scaled up to large fermenters in which a uniform distribution
815 of carbon source, oxygen and pH is difficult to achieve. Glycoside hydrolases can potentially be
816 commercialized for large-scale applications, making them a better option than
817 glycosyltransferases, which are difficult to express and need expensive substrates. In particular, β-
818 galactosidases with a suitable transgalactosylation activity for catalyzing such reactions are
819 commercially available. While these enzymes have been predominantly investigated in GOS
820 production processes, β-galactosidases can also catalyze the production of some important lactose-
821 derived molecules, such as *N*-acetyllactosamine and lacto-*N*-neotetraose. The main drawback of
822 transglycosylation using glycoside hydrolases is the undesirable hydrolysis reaction, which
823 simultaneously occurs alongside transglycosylation. Nonetheless, these reactions can be regulated
824 by controlling factors such as temperature, water activity and substrate concentration, and not least
825 protein engineering of the enzymes as exemplified in this review.

Due to the significant progress in protein expression and engineering, both simple and complex LacNAc glycans have recently been successfully synthesized in the laboratory. The scalability of such reactions is dependent on the availability of commercial enzymes (or contract manufacturing of enzymes), inexpensive starting materials and the number of upstream and downstream operations, which can affect the overall process time and complexity. To reduce the process costs, the number of enzymes involved in multi-step enzymatic reactions should be kept to a minimum; this could be achieved through combining glycosidase-catalyzed or microbial synthesis routes sourced with inexpensive substrates with transferase-catalyzed reactions to obtain LacNAc core structures needed for further branched LacNAc glycans. The application of regeneration systems is another strategy to streamline large-scale production; however, not all these systems are cost-effective unless further techno-economic studies validate their scalability. Regeneration systems with inexpensive starting materials, a low number of cofactor requirements and the use of immobilised enzymes to facilitate a large number of regeneration cycles will be more viable to progress beyond a discovery scale.

Given the current growth of the global bioactive ingredient market and the vast diversity of such LacNAc-based bioactive compounds, we find that further focus on robust enzyme development and reaction technology optimization are imperative for successful large-scale production.

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Figure Captions

Figure 1. Structures of *N*-acetyllactosamine (LacNAc), Lacto-*N*-neotetraose (LNnT), lacto-*N*-triose II (LNT2), 6'sialyl LacNAc, 3'sialyl LacNAc, poly-LacNAc, P1 antigen, 6'sialyl LacNAc-GLP-1, LacNAc-superparamagnetic iron oxide nanoparticles (SPIONs) and 6'sialyl LacNAc-lipo PGA.

Figure 2. Heyns rearrangement of lactulose for synthesis of LacNAc.[57] Reagents and conditions: (i) Benzylamine, 40°C, 3 d; (ii) Methanol, glacial acetic acid, 2 h; (iii) HCl, pH 1–2, $Pd(OH)_2/C$, H_2, 6 bar, 40°C; (iv) Methanol, Sodium methoxide, Acetic anhydride, 40°C.

Figure 3. The linear synthesis of Lacto-*N*-neotetraose (LNnT) (**10**) from a glucosamine thioglycoside precursor (**1**) and a lactose precursor (**4**).[61] Reagents and conditions: (i) FmocCl, Py, CH_2Cl_2, 2 h; (ii) $HOPO(OBu)_2$ NIS/TtOH, CH2Cl2, molecular sieves 3 Å, 0 °C, 20 min, 90%; (iii) TMSOTf, CH_2Cl_2, molecular sieves 3 Å, -30 ºC, 15 min, 70%; (iv) 30% Et_3N/CH_2Cl_2, 2 h, 98%; (v) AgOTf, CH_2Cl_2, molecular sieves 3 Å, -30 ºC, 15 min, 84%; (vi) $NH_2.NH_2.H_2O$, Methanol, 80 ºC, 24 h followed by Ac_2O, Methanol, 16 h, 87%; (vii) Pd/C, H_2, EtOH, 16 h, 92%. FmocCl: fluorenylmethyloxycarbonyl chloride, Et_3N: Triethylamine; NIS: N-iodosuccinimide; TfOH: triflic acid; TMSOTf: trimethylsilyl trifluoromethanesulfonate; AgOTf: silver trifluoromethanesulfonate; Ac_2O: acetic anhydride.

Figure 4. (A) Coupled microbial synthesis of LacNAc via the combination of two recombinant *Escherichia coli* strains (*E. coli* NM522/pGT8 and *E. coli* NM522/pNT2/pNT32) and *Corynebacterium ammoniagenes* DN510. *E. coli* NM522/pNT2/pNT32 and *C. ammoniagenes* DN510 are attributed to the UDP production system. *E. coli* NM522/pGT8 expressing a β1-4 galactosyltransferase gene of *Neisseria gonorrhoeae* produces LacNAc from UDP-Gal. *ppa*:

pyrophosphatase, *galK*: galactokinase, *lgtB*: β1-4 galactosyltransferase gene of *N. gonorrhoeae* F62.[81] (B) Synthesis of lacto-*N*-triose II (LNT2) from lactose using a genetically modified *E. coli* strain expressing the *N. meningitidis* β1-3 *N*-acetylglucosaminyltransferase *lgtA* gene. (C) Synthesis of Lacto-*N*-neotetraose (LNnT) from lactose by co-expression of *N. meningitidis* β1-4 galactosyltransferase *lgtB* gene with *lgtA*. Lactose transported to the *E. coli* cells by β-galactoside permease LacY is not hydrolyzed to glucose and galactose because of the lack of the LacZ gene. LNT2 is released to the extracellular medium while LNnT remains intracellular.[78]

Figure 5. Core LacNAc-containing oligosaccharides that can be obtained from lactose using glycosyltransferases. LacNAc moieties are highlighted in green. iGnT: β1-3 *N*-acetylglucosaminyltransferase, IGnT: β1,6 *N*-acetylglucosaminyltransferase, β3GalT: β1-3 galactosyltransferase, β4GalT: β1-4 galactosyltransferase, LNT: Lacto-*N*-tetraose, LNnT: Lacto-*N*-neotetraose, *p*LNH: *para*-Lacto-*N*-hexaose, *p*LNnH: *para*-Lacto-*N*-neo-hexaose, *p*LNO: *para*-Lacto-*N*-octaose, LNnH: Lacto-*N*-neo-hexaose, LNH: Lacto-*N*-hexaose, *i*LNO: *iso*-Lacto-*N*-octaose, LND: Lacto-*N*-decaose, LNnD: Lacto-*N*-neo-decaose, LNnO: Lacto-*N*-neo-octaose, LNO: Lacto-*N*-octaose.[218]

Figure 6. (A) LacNAc synthesis by *in situ* regeneration of the nucleotide sugar UDP-Glc and UDP-Gal from sucrose using a sucrose synthase from rice grains, a UDP-Glc 4'-epimerase from *Saccharomyces cerevisiae* and a β1-4 galactosyltransferase from human milk. (B) Repetitive-batch technique for enhancing enzyme productivity.[105]

Figure 7. Synthesis of 6’ sialyl LacNAc from cytidine-5'-monophosphate-5-*N*-acetylneuraminic acid (CMP-Neu5Ac) as donor and LacNAc as acceptor using an α2-6 siyalyltransferase (EC 2.4.99.1) with *in situ* generation of *N*-acetylneuraminic acid from *N*-acetylmannosamine

(ManNAc) and pyruvate catalyzed by a sialyl aldolase (EC 4.1.3.3) associated with *in situ* regeneration of CMP-Neu5Ac catalyzed by a CMP-Neu5Ac synthase (EC 2.7.7.43).[110]

Figure 8. Synthesis of LacNAc-ITag from GlcNAc-ITag and UDP-Gal (Pathway 1) or from GlcNAc-ITag and lactose (Lac) in the presence of UDP (Pathway 2) using a β1-4 galactosyltransferases (β4GalT) from *Neisseria meningitidis* serogroup B strain MC58 (NmLgtB-B). (B) Synthesis of *p*NP-β-LacNAc from *p*NP-β-GlcNAc and lactose in the presence of UDP using the β4GalT.[73]

Figure 9. (A) Reaction mechanism and possible pathways included in β-galactosidase-catalyzed reactions.[219] Structures of (B) *N*-acetylneuraminic acid (Neu5Ac), (C) Neu5Ac in an α2-6-linkage to galactose and (D) Neu5Ac in an α2-3-linkage to galactose.[220] (E) Reaction mechanism and pathways included in sialidase-catalyzed reactions.[129]

Figure 10. β-*N*-Acetylhexosaminidase-catalyzed transgalactosylation of lactose for the synthesis of LNT2 with different GlcNAc containing donors; followed by β-galactosidase-catalyzed transgalactosylation of LNT2 with lactose as donor for the synthesis of LNnT.