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

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13 **Keywords:** Lactose; Whey; β -Galactosidases; Sialidases; β -*N*-Acetylhexosaminidases;

14 Glycosyltransferases.

15

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16 **Abstract**

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

39 **Abbreviations:**

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

41 INTRODUCTION

42 To date, over 150 different human milk oligosaccharide (HMO) structures have been
43 discovered.¹ The focus of this review is *N*-acetylglucosamine (LacNAc; *N*-acetyl-4-*O*-(β -D-
44 galactopyranosyl)-D-glucosamine or β -D-galactopyranosyl-1,4-*N*-acetyl-D-glucosamine; Figure 1),
45 which is a core structure in many of these HMOs, including lacto-*N*-neotetraose (LNnT; LacNAc-
46 β 1-3-Lactose), a series of lacto-*N*-octaose HMOs, sialyllacto-*N*-neotetraose c (LSTc) and
47 monofucosyllacto-*N*-hexaose (MFLNH).²⁻³ LNnT (Figure 1) is of particular note, as it is
48 recommended for the manufacture of safe, well-tolerated, age-appropriate infant formula⁴ and is
49 available in more than 30 countries within such products.⁵⁻⁶ Commercial-scale synthesis of these
50 HMOs, however, is limited.⁵

51 **(Figure 1)**

52 Some more complex HMO structures can be obtained by elongating other core structures
53 or precursors.⁷⁻⁸ Poly-*N*-acetylglucosamine (poly-LacNAc; Figure 1) structures are not HMOs but
54 have been investigated in a number of studies, due to their essential biological roles in cell-cell
55 interactions, tumor progression and immune response.⁹⁻¹¹ These structures are found on the surface
56 of a range of animal cells including rat, rabbit, human erythrocytes and human leukocytes.¹⁰ Poly-
57 LacNAc structures can be synthesized enzymatically using either LacNAc and LNnT.¹²⁻¹³ As
58 LacNAc and poly-LacNAc can be used as specific lectin target molecules and bind to the galectin
59 carbohydrate recognition site, they are also useful biomarkers for the detection of cancer cells.^{11,}
60 ¹⁴⁻¹⁹

61 LacNAc can also act as a precursor for many other biological compounds, namely
62 glycolipids,²⁰⁻²¹ glycoproteins^{20, 22} and some red blood cell components, including the Lewis and

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

71 LacNAc has a number of potentially beneficial physiological effects and appears to work
72 through a variety of mechanisms. Hyland et al.²⁸ showed that LacNAc glycosides conjugated to
73 BSA (LacNAc-BSA) or gold nanoparticles (LacNAc-Au) inhibited the localized adherence of
74 enteropathogenic *Escherichia coli* (EPEC) to human epithelial type 2 (HEp-2) cells. LacNAc may
75 also assist drug stability, for example glucagon-like peptide 1 (GLP-1), which is proposed for the
76 treatment of type 2 diabetes.²⁹⁻³⁰ The *in vivo* use of this hormone is restricted due to its physical
77 instability and aggregation, short half-life in plasma and inactivation by proteolytic enzymes.³¹⁻³²
78 Ueda et al.³³ demonstrated that the addition of LacNAc and α 2-6-sialyl-N-acetyllactosamine
79 (6'-sialyl LacNAc or 6'-SLN) to GLP-1 (Figure 1) could enhance this proteolytic stability and thus
80 the *in vivo* blood glucose-lowering activity of GLP-1. Given the broad potential of glycosylation
81 to increase stability, to enhance activity, to alter tissue specificity, reduce toxicity and alter blood
82 brain permeability,³⁴⁻³⁶ there may be much broader potential for LacNAc across a range of
83 pharmaceutical applications.

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

86 (Gd³⁺) complexes are commonly used as MRI contrast agents to enhance the quality of these
87 images but the release of Gd³⁺ in the human body is of major concern given Gd³⁺ toxicity.³⁷⁻³⁸
88 Recently, superparamagnetic iron oxide nanoparticles (SPIONs) coated with LacNAc (Figure 1)
89 have been developed as an alternative MRI contrast agent, which is more biocompatible with
90 human body fluids.³⁹

91 The possible application of LacNAc-based molecules in antiviral drugs has also been
92 emphasized in several studies. Influenza viruses are constantly changing and the evolution of
93 mutant viruses spread through human-to-human contact can have fatal consequences.⁴⁰⁻⁴¹
94 Currently, the number of humans and poultry vaccinated each year is insufficient for the prevention
95 of an outbreak.⁴²⁻⁴³ Wang et al.⁴⁴ proposed that antiviral drugs based on sialylated LacNAc
96 molecules (3'-SLN and 6'-SLN; Figure 1) may be effective against all mutant viruses, as these
97 molecules are the cell receptors for the hemagglutinin binding of influenza viruses to the
98 epithelium within the human respiratory tract. In a recent study by Kocabiyik et al.⁴⁵ a new class
99 of anti-influenza macromolecules based on a cyclodextrin core and 6'-SLN or 3'-SLN terminals
100 were described. They showed that these antiviral drugs can suppress several human or avian
101 influenza strains *in vitro*, as well as a 2009 pandemic influenza strain *ex vivo* and *in vivo*. Likewise,
102 Sriwilaijaroen et al.⁴⁶ developed a new drug based on 6'-SLN (6'-SLN-lipo PGA; Figure 1) for
103 eradication of human influenza. This mechanism may also be useful across species, as it has been
104 shown that Guinea fowl coronavirus (GfCoV) can bind to di-*N*-acetyllactosamine and glycans
105 capped with α 2-6-linked sialic acids.⁴⁷ A further example is the Middle East respiratory syndrome
106 coronavirus (MERS-CoV), as no vaccine or specific treatment is currently available for this virus,
107 which results in an acute respiratory infection in humans following attachment of the virus to
108 human cells via a spike protein. Park et al.⁴⁸ recently investigated the attachment of MERS-CoV

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

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

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

132 with an 85% purity was achieved after several purification steps, this mixture only contained 17%
133 6'-SLN as the LacNAc-based saccharide.⁵³ Thus, synthetic approaches towards target *N*-
134 acetyllactosamine related structures have attracted widespread attention.

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

140 **CHEMICAL SYNTHESIS**

141 LacNAc can be produced through a series of chemical reactions.⁵⁴⁻⁵⁵ These methods are,
142 however, not suitable for industrial production due to the use of toxic reagents, such as hydrogen
143 cyanide, as well as the production of many intermediates; low stereoselectivity and the presence
144 of epimers; as well as low overall yields. In a study by Lattová and Petruš⁵⁶ an ozonolysis reaction
145 was employed to produce LacNAc (32% yield) from 3-*O*- β -D-galactopyranosyl-D-arabinose.
146 Likewise, 2-acetamido-2-deoxy-4-*O*- β -D-galactopyranosyl-D-mannopyranose (a LacNAc epimer)
147 was obtained with a yield of 15%.

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

154 temperature. In the next step, hydrogenolysis of *N*-benzyl lactosamine to D-lactosamine
155 hydrochloride was conducted using hydrogen in the presence of HCl and palladium hydroxide on
156 carbon (Pd(OH)₂/C). The crude D-lactosamine hydrochloride was then *N*-acetylated by adding
157 sodium methoxide and subsequently acetic anhydride to give LacNAc. A total yield of 48% on a
158 25 g scale was achieved after peracetylation, using acetic anhydride and chromatographic
159 separation. Although commercially available lactulose can be used in this reaction, with relatively
160 higher yields, it requires several reagents and the product isolation is also problematic.

161 **(Figure 2)**

162 More complex chemical routes have also been developed for the synthesis of LacNAc
163 derivatives. Lemieux et al.⁵⁹ synthesized a LacNAc derivative, hexa-*O*-acetyl-2-deoxy-2-
164 phthalimido-β-D-lactosyl chloride, from D-lactal hexaacetate via a seven-stage azido-nitration
165 route. In a study by Malet and Hindsgaul⁶⁰ 24 LacNAc derivatives carrying carboxymethyl,
166 amidinomethyl, aminoethyl and carbamoylmethyl substituents were chemically generated.

167 Bandara et al.⁶¹ developed a linear synthetic approach for the chemical synthesis of LNnT as shown
168 in Figure 3. They synthesized a glucosamine thioglycoside precursor (**1**) based on a seven-step
169 process previously developed by Nagorny et al.⁶² This precursor was then reacted with
170 fluorenylmethyloxycarbonyl chloride (FmocCl) in the presence of pyridine in CH₂Cl₂ to obtain its
171 Fmoc derivative (**2**) which was subsequently converted to a phosphate donor (**3**) in the presence
172 of dibutyl phosphate, freshly activated molecular sieves (3 Å) in CH₂Cl₂, *N*-iodosuccinimide and
173 triflic acid. A lactose precursor (**4**) was synthesized via the coupling between an orthogonally
174 protected galactose donor and a tetrabenzylated glucose 4-OH acceptor in a series of chemical
175 reactions previously reported.⁶³⁻⁶⁴ A trisaccharide (**5**) was then produced via glycosylation between

176 the phosphate donor (3) and the lactose acceptor (4) in the presence of trimethylsilyl
177 trifluoromethanesulfonate. Triethylamine in CH₂Cl₂ was then used to remove the Fmoc protecting
178 group to achieve a trisaccharide acceptor (6). The trisaccharide acceptor obtained (6) was reacted
179 with a *S*-benzoxazolyl donor (7) in the presence of silver trifluoromethanesulfonate to produce a
180 β-linked tetrasaccharide intermediate (8). The intermediate (8) was deprotonated in two steps
181 including refluxing with NH₂NH₂-H₂O in MeOH followed by acetic anhydride in MeOH
182 treatment. The resulting *N*-acetylated tetrasaccharide (9) was treated with 10% palladium on
183 charcoal in wet ethanol to eliminate benzyl groups, resulting in the target LNnT product (10) with
184 an overall yield of 57%. Likewise, several poly-LacNAc structures have been synthesized through
185 a complicated series of chemical reactions.⁶⁵⁻⁷⁰ The many steps required, however, represent a
186 barrier for these processes being commercially competitive.

187 **(Figure 3)**

188 **BIOCHEMICAL SYNTHESIS**

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

195 The use of cheaper substrates for such biochemical synthesis could facilitate commercial
196 production. Lactose is an abundant and inexpensive substrate for the synthesis of value-added
197 functional food products⁷⁵⁻⁷⁶ and can be utilized in both *in vivo* and *in vitro* biochemical approaches

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

203 **Microbial production**

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

213 Endo et al.⁸¹ developed a coupled microbial system containing two recombinant
214 *Escherichia coli* strains and *Corynebacterium ammoniagenes* for the production of LacNAc from
215 UDP-Gal (uridine-5'-diphosphate galactose) (Figure 4(A)). The UDP-Gal production was
216 performed by a recombinant *E. coli* expressing the UDP-Gal biosynthetic genes (*E. coli*
217 NM522/pNT2/pNT32) and *C. ammoniagenes* DN510 forming UTP from orotic acid. The reaction
218 mixture contained *E. coli* NM522/pGT8 cells expressing the β 1-4 galactosyltransferase gene of
219 *Neisseria gonorrhoeae*, *E. coli* NM522/pNT2/pNT32 and *C. ammoniagenes* DN510 cells. Orotic

220 acid, GlcNAc, as well as galactose, were added to the reaction mixture. It was shown that GlcNAc
221 is transported to *E. coli* strains by the action of nag genes.⁸²⁻⁸³ After 38 hours, these cells produced
222 107 g l⁻¹ (279 mM) of LacNAc from 578 mM galactose and 290 mM GlcNAc, giving a molar yield
223 of 48% based on galactose and 96% based on GlcNAc. Despite these high yields, the long reaction
224 times (38 hours) led to a lower productivity and may potentially restrict commercialization.

225 A metabolically engineered *E. coli* strain (*E. coli* JM109) lacking the β -galactosidase gene
226 (*lacZ*) was also used for the effective synthesis of lacto-*N*-triose II (LNT2).⁷⁸ As shown in Figure
227 4(B), the β -galactoside permease LacY transported lactose into the cell but due to the removal of
228 hydrolytic activity (deletion of the *lacZ* gene), lactose was not converted to glucose and galactose.
229 Under these conditions LNT2 (6 g l⁻¹) was obtained, due to the expression of a *lgtA* gene of
230 *Neisseria meningitidis* encoding a β 1-3 *N*-acetylglucosaminyltransferase with the use of an
231 endogenous UDP-*N*-acetylglucosamine. The cultivation of these engineered bacteria at high
232 densities was performed using glycerol as the carbon and energy source. LNnT was produced,
233 together with other poly-LacNAc structures, including lacto-*N*-neohexaose (LNnH), lacto-*N*-
234 neo-octaose (LNnO) and lacto-*N*-neodecaose (LNnD) due to the co-expression of the *lgtB* gene
235 encoding a β 1-4 galactosyltransferase with the *lgtA* gene in the JM109 strain. In this case, the strain
236 JM109 was grown on a glucose medium instead of glycerol owing to the toxicity resulting from
237 the overexpression of the two genes on a glycerol medium. The target oligosaccharides
238 accumulated inside the cells and were not released to the extracellular medium due to their large
239 sizes. Therefore, these intracellular products were purified by charcoal adsorption during
240 subsequent purification of the LNT2 product.

241 **(Figure 4)**

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

247 No wild type microorganism has been reported to generate LacNAc-derived structures
248 naturally, indicating that the need for genetic modification is inevitable⁸⁵ Microbial production of
249 more complex oligosaccharides necessitates the expression of further genes⁸⁴ and optimization of
250 these systems for high productivity. The disadvantage of this approach, employing whole cells, is
251 that these systems can be difficult to control and optimize, with undesirable side reactions, product
252 or substrate inhibitory effects, limitations in the uptake of high concentrations of substrates and
253 complex culture media, requiring research to optimize these systems. In addition, large fermenters
254 (over 200 m³) generate gradients of carbon source, oxygen and pH, making the control of carbon
255 source feeding rate, as well as prevention of overflow or anerobic metabolism more challenging.
256 In addition, the formation of inhibitory metabolites and environmental stresses can lead to lower
257 biomass yields.⁵ Whole cell systems are therefore typically employed when numerous enzymes in
258 multi-step reactions are required and an engineered host cell can offer advantages or when enzyme
259 production and isolation is difficult and costly.^{5, 86-88}

260 ***In vitro* enzymatic synthesis**

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

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

272 **Glycosyltransferases for synthesis of LacNAc-based molecules**

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

280 Based on the type of linkage formed and substrate specificity, different families of
281 galactosyltransferases have been identified. β 1-3 galactosyltransferase (β 3GalT; GT31) genes are
282 responsible for the expression of enzymes transferring Gal unit to the β 1-3 position of GlcNAc,⁹⁹
283 with the Gal β 1 \rightarrow 4GlcNAc (LacNAc) moiety being prevalent in many oligosaccharides. β 1-4
284 galactosyltransferase (β 4GalT; GT7) enzymes catalyze the transfer of galactose from UDP-Gal to
285 GlcNAc.¹⁰⁰ The transfer of GlcNAc from UDP-GlcNAc to Gal is also directed by β 1-3 *N*-

286 acetylglucosaminyltransferase (iGnT; GT14). Various core LacNAc-containing oligosaccharides
287 can also be synthesized by β 4GalT and iGnT starting from lactose (Figure 5).

288 **(Figure 5)**

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

303 The product yield can be high in reactions catalyzed by such glycosyltransferases. For
304 example, a mixture of enzymes including a galactosyltransferase (EC 2.4.1.22) and accompanying
305 enzymes, normally known as ground working enzymes, (UDP-galactose 4'-epimerase (EC
306 5.1.3.2), UDP-glucose pyrophosphorylase (EC 2.3.7.9), phosphoglucomutase (EC 2.7.5.1) and
307 pyruvate kinase (EC 2.7.1.40)) using different substrates, including glucose 6-phosphate (50 mM),

308 UDP (0.625 mM), GlcNAc (50 mM) and phosphoenolpyruvate (52.5 mM), resulted in a molar
309 yield of 85% LacNAc based on glucose 6-phosphate after 96 hours at room temperature, followed
310 by treatment with ion exchange resins.¹⁰³ The isolation yield after further purification by gel
311 permeation chromatography was 70%. In another example, Yu et al.¹⁰⁴ developed a sequential one-
312 pot multi-enzyme synthesis of LNT2 (isolation yield of 95%) and LNnT (isolation yield of 92%)
313 from lactose. The products obtained were further elongated to two sialylated HMOs, α 2-6-linked
314 disialyllacto-*N*-neotetraose (DSLNT) (isolation yield 99%) and α 2-6-linked disialyllacto-*N*-
315 tetraose (DS'LNT) with an isolation yield of 98%. This multi-enzyme system included several
316 types of glycosyltransferases, namely *N*-acetylglucosaminyltransferase from *Pasteurella*
317 *multocida*, β 1-3 *N*-acetylglucosaminyltransferase from *Neisseria meningitidis*, β 1-4
318 galactosyltransferase from *Neisseria meningitidis* and α 2-6 sialyltransferase from *Photobacterium*
319 *damselae*. Chen et al.¹² developed an enzymatic modular assembly with the aid of bacterial
320 glycosyltransferases, including β 1-3 *N*-acetylglucosaminylation (*Helicobacter pylori*), β 1-4
321 galactosylation (*Neisseria meningitidis*), α 1,3 fucosylation (*Helicobacter pylori*) and α 2-3
322 sialylation (*Pasteurella multocida*), for the synthesis of thirty three poly-LacNAc derivatives
323 starting from LNnT. In most cases, the oligosaccharide yields were more than 90%, illustrating
324 the potential of this approach.

325 The key issue and the potential barrier for large-scale production using glycosyltransferases is
326 the requirement for regeneration of the sugar nucleotide glycosyl donor. One potential solution to
327 this barrier is the production of LacNAc with *in situ* regeneration of uridine-5'-diphosphate glucose
328 (UDP-Glc) and uridine-5'-diphosphate galactose (UDP-Gal).¹⁰⁵ A system of three enzymes,
329 sucrose synthase (from rice grains), UDP-Glc 4'-epimerase (from *Saccharomyces cerevisiae*) and
330 β 1-4 galactosyltransferase (from human milk), was used with a complex mixture of different

331 substrates including sucrose (500 mM), UDP-Glc (10 mM), GlcNAc (100 mM) and dUDP or
332 dTDP-6-deoxy-D-xylo-4-hexulose (1 mM) for the synthesis of around 350 mg LacNAc (Figure
333 6(A)). A multistep isolation method was then employed to separate the components in the reaction
334 medium. This commenced with enzymatic cleavage of sucrose to glucose and fructose using
335 invertase from yeast; and continued with separation of LacNAc from monosaccharides by ligand
336 exchange chromatography with a cation exchanger in Ca²⁺-form; anion-exchange chromatography
337 for the removal of nucleotides, nucleotide sugars, as well as buffer salt; and was completed with
338 gel filtration for the removal of residual glucose and GlcNAc. To reduce the cost of the enzymes
339 and improve productivity, ultrafiltration was used to separate the product from the enzyme, which
340 was retained in the reaction medium (Figure 6(B)). Fresh substrate was then added and the method
341 repeated over 11 days in 11 batches. While the LacNAc yield obtained after 22 hours was almost
342 100% in the first batch, it decreased to 40-50% with a longer incubation time (30 hours) after 11
343 batches due to inactivation of the enzyme during the synthesis and filtration. The average LacNAc
344 yield was 57.4%. Transition-state analogs dUDP- or dTDP-6-deoxy-D-xylo-4-hexulose were also
345 added to the mixture to reactivate the UDP-glucose 4'-epimerase.

346 **(Figure 6)**

347 In another substrate regeneration study by Chen et al.¹⁰⁶ a system of multiple immobilized
348 enzymes was created for UDP-Gal regeneration. Four enzymes: galactokinase (GalK; EC 2.7.1.6),
349 galactose-1-phosphate uridylyltransferase (GalPUT; EC 2.7.7.12), glucose-1-phosphate
350 uridylyltransferase (GalU; EC 2.7.7.9) and pyruvate kinase (PykF; EC 2.7.1.40) were
351 immobilized onto Ni²⁺-NTA agarose beads. The beads were then incubated with a bovine β 1-4
352 galactosyltransferase and a mixture of GlcNAc (0.24 mmol), ATP (24 μ mol),
353 phosphoenolpyruvate (0.48 mmol), UDP (24 μ mol), glucose-1-phosphate (24 μ mol) and galactose

354 (0.3 mmol). After reaction, the beads were separated and the LacNAc product was purified using
355 anion exchange followed by gel permeation chromatography. The total amount of LacNAc
356 obtained was 85 mg (0.22 mmol) giving a yield of 92%. This regeneration system enables the
357 reuse of the enzymes but the long reaction time (four days) might hamper large-scale synthesis.

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

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

377 monophosphate kinase, pyrophosphorylase, sialyl aldolase, CMP-Neu5Ac synthase and α 2-6
378 sialyltransferase were added to the reaction buffer (HEPES; 200 mM, pH 7.5). The reaction was
379 performed at room temperature for two days.

380 **(Figure 7)**

381 The exploitation of glycosyltransferases as tools for the synthesis of complex LacNAc-based
382 extensions has been studied by several leading research groups. Paulson and co-workers.¹¹¹
383 reported the enzymatic synthesis of multi-sialylated poly-LacNAc glycans using a recombinant
384 α 2-6 sialyltransferase from *Photobacterium damsela* (Pd2,6ST) and CMP-Neu5Ac as the donor
385 substrate. They synthesized different oligo- and poly-LacNAc acceptors based on previously
386 developed glycosyltransferase-based methods.¹¹²⁻¹¹³ These methods are of significant importance,
387 particularly for the production of such poly-LacNAc extensions in quantities needed for detailed
388 biological studies and the generation of glycan libraries. Nonetheless, further research is still
389 needed to commercialize the advanced core LacNAc structures needed for elongation of poly-
390 LacNAc chains in glycosyltransferase-catalyzed cascade reactions.

391 In a similar study by Lin and co-workers¹¹⁴ a sequential enzymatic process was developed to
392 synthesize oligo-LacNAcs followed by sialylation of oligo-LacNAcs at a milligram scale. They
393 first synthesized LacNAc (46 mg) using a 6-azidohexyl-GlcNAc (azido-GlcNAc) acceptor and a
394 UDP-Gal donor in a reaction catalyzed by a recombinant β 1-4 galactosyltransferase from *Neisseria*
395 *meningitidis*. The one-pot synthesis of UDP-Gal from galactose-1-phosphate (Gal-1-P) was
396 performed by a thermophilic thymidyltransferase (RmlA) from *Aneurinibacillus*
397 *thermoaerophilus* coupled with a recombinant galactokinase (MtGalK) from *Meiothermus*
398 *taiwanensis*. A β 1,3-*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 α 2-6 sialyltransferase (Pd2,6ST from *Photobacterium*
405 *damselae*) or an α 2-3 sialyltransferase (PmST from *Pasteurella multocida*).

406 Yu and co-workers⁷ 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-workers¹¹⁴ 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 β 1,3 *N*-
411 acetylglucosaminyltransferase from *H. pylori* strain 26695 (HP1105). A β 1,4 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 β 1,3
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-workers⁷ described above, the KH-1 antigen has recently been produced by the same
421 group¹¹⁵ via multi-fucosylation of *p*-LNnH in a sequential enzymatic reaction. They reacted 20 mg

422 of *p*-LNnH with GDP-Fuc by an α 1,2 fucosyltransferase from *H. pylori* (HpFutC) for 3 hours to
423 obtain branched fucosyl *para*-lacto-*N*-hexaose (F-*p*LNnH). After the reaction, a fucosyltransferase
424 from *Bacteroides fragilis* NCTC 9343 (Bf13FT) was added to the solution containing F-*p*LNnH.
425 After 6 hours, a mixture of fucosylated products including two inseparable difucosylated isomers
426 (0.5 mg), the KH-1 antigen (16.2 mg) and one tetra-fucosylated product (6.6 mg) was obtained.
427 Although the overall sequential reaction time was 103 hours, this study demonstrates the potential
428 importance of bacterial glycosyltrasferases for the synthesis of such complex LacNAc-based
429 structures.

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

442 **(Figure 8)**

443

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

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

462

463 **Glycoside hydrolases for synthesis of LacNAc-based molecules**

464 **β -Galactosidases**

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

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

491 **Table 1.** Synthesis of LacNAc from lactose or phenyl-D-galactopyranosides as donors and
 492 GlcNAc as acceptor, using β -galactosidase enzymes.
 493

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

ⁱYield based on HPLC analysis of crude product, unless specified as the yield after isolation.

ⁱⁱThe LacNAc produced was calculated based on the reaction volume where available. na: not available

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

501 **Substrate concentration and specificity**

502 It has been reported that the initial substrate concentration in enzymatic transglycosylation
503 reactions can affect the product yield.¹⁴⁰⁻¹⁴¹ In addition, the initial substrate concentration may
504 change the product composition.¹⁴² Li et al.¹³¹ showed that at lactose concentrations greater than
505 1 M, the yield of LacNAc decreased, while the production of Allo-LacNAc increased with the
506 increase of substrate concentration. These effects may be enzyme specific, however, as it was also
507 concluded that β -galactosidase from *L. bulgaricus* was not inhibited by its substrates, i.e. lactose
508 at concentrations up to 600 mM or 2-nitrophenyl β -D-galactopyranoside (*o*NPG) at concentrations
509 up to 25 mM.¹⁴³ Furthermore, many β -galactosidases show different affinities to various
510 substrates. These enzymes are specific to the β -D-glycosidic linkage and display a better specificity
511 for galactosides containing *ortho*- or *para*-nitrophenyl groups rather than methyl or phenyl
512 groups.¹⁴⁴⁻¹⁴⁵

513 **Cations**

514 Whey is a source of lactose that can be used directly for performing an *in situ*
515 transgalactosylation reaction. The existence of cations, namely sodium, potassium, magnesium
516 and calcium in the whey, however, may affect the enzymatic reaction. Depending on the
517 biocatalyst source, cations may affect the hydrolytic activity in different ways. For example,
518 divalent cations, including Mn^{2+} , Co^{2+} , Fe^{2+} , Mg^{2+} and Ca^{2+} , enhanced the hydrolytic activity of a
519 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 Kleinschmidt¹⁴⁸ 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

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Water activity

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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.

561

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 (Tt β -gly) and *Pyrococcus furiosus* (CelB) for the production of
565 LNT 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 Tt β -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 Tt β -gly and CelB, respectively. Working at an elevated temperature, however, improved the yield
570 of LacNAc by 5 times (BgaD-D) and 6 times (Tt β -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 Adlercreutz¹⁵¹
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

581 **Sialidases**

582 Sialic acids are another interesting functionality that may be transferred onto LacNAc-
583 based structures. The biological functions of numerous sialo-glycoconjugates on the human cell
584 surface are controlled by sialic acids, including *N*-acetylneuraminic acid (Neu5Ac), deaminated
585 neuraminic acid (KDN), *N*-glycolylneuraminic acid (Neu5Gc) and neuraminic acid (Neu), with
586 around 50 sialic acid derivatives discovered to date. The majority of these are Neu5Ac in an α 2-3-
587 or α 2-6-linkage to galactose (Gal) (Figure 9(B-D)).^{80, 152} There are also other dominant structures
588 including an α 2-3- or α 2-6-linkage to *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine
589 (GlcNAc) or an α 2-8-linkage to another *N*-acetylneuraminic acid at the terminal position of
590 glycans.¹⁵³

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

602 Sialidases with disparate kinetics, substrate specificity and regioselectivity can be obtained
603 from various bacterial strains, including *Arthrobacter nicotianae*, *Arthrobacter ureafaciens*,
604 *Clostridium perfringens*, *Pasteurella multocida* and *Streptococcus pneumoniae*.¹⁵⁷ Displaying a
605 high trans-sialylation activity, sialidases from *Trypanosoma* species, such as *Trypanosoma cruzi*,
606 *Trypanosoma congolense* and *Trypanosoma brucei*, have substrate specificities to different
607 acceptors. The sialidase from *T. cruzi* can only act on substrates with the α 2-3-linked sialyl
608 residues,¹⁵⁸ while the sialidase from *Arthrobacter sialophilus* is specific for either of the α 2-3-, α 2-
609 6- or α 2-8-linked sialic acids.¹⁵⁷ Sialidases from *Clostridium perfringens*, *Arthrobacter*
610 *ureafaciens*, *Vibrio cholerae* and *Newcastle disease virus* can also hydrolyze α 2-8-linked sialic
611 acids.¹²⁹ Compared to sialyltransferases, the yield of sialylation product is low (generally <30%)
612 for most sialidases derived from different sources. The trans-sialidase from *T. cruzi*, however, has
613 been shown to have much higher yields (up to 80%) depending on the donor substrate.^{129, 159-160}

614 Scudder et al.¹⁶¹ reported that *T. cruzi* trans-sialidase is able to sialylate a wide range of
615 substrates with subterminal β 1-4Man, β 1-4Gal, β 1-6Gal and β 1-6GlcNAc as well as LacNAc,
616 LNnT, LNT and lacto-*N*-fucopentaose V (LNFP V). They showed that starting with LNT as the
617 acceptor substrate and sialyllactose as the sialyl donor, *T. cruzi* trans-sialidase converted 66% of
618 LNT to a precursor of sialyl-Lewis^x and sialyl-Lewis^a. Michalak et al.¹⁶² successfully modified a
619 sialidase derived from *Trypanosoma rangeli* with superior trans-sialidase activity. They expressed
620 the enzyme in *Pichia pastoris* and then used it for sialylation of LNT, LNnT, lacto-*N*-fucopentaose
621 I (LNFP I) and LNFPV. A total of 3.6 g 3'-sialyllactose was synthesized in a pilot scale (5 l) reactor
622 using CGMP as the sialyl donor and lactose as the acceptor after 20 min. Trans-sialylation of LNT,
623 LNnT, LNFP I and LNFPV as the acceptors and CGMP as the donor was performed in a 5-100 ml
624 scale reactor which resulted in 5-20 mg of sialylated oligosaccharides. These examples are

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

628 **β -N-Acetylhexosaminidases**

629 Some extended LacNAc-core structures can be enzymatically synthesized by the action of a
630 group of glycoside hydrolases known as β -N-acetylhexosaminidases. β -N-Acetylhexosaminidases
631 are GH20 glycoside hydrolases that catalyse cleavage of terminal, non-reducing N-
632 acetylhexosamine moieties and can catalyze the hydrolysis of N-acetylglucosides or N-
633 acetylgalactosides containing a 2-acetamido group.¹⁶³⁻¹⁶⁴ They have attracted attention due to their
634 inherent ability to catalyze transglycosylation reactions in which N-acetylhexosamine residues,
635 namely GlcNAc or GalNAc, are transferred from a donor substrate to an acceptor substrate,
636 creating more complex bioactive carbohydrates.¹⁶⁵⁻¹⁶⁹ Artificial *para*-nitrophenyl donors including
637 *para*-nitrophenyl N-acetyl- β -D-glucosaminide (*p*NP- β -GlcNAc) and *para*-nitrophenyl N-acetyl- β -
638 D-galactosaminide (*p*NP- β -GalNAc) have been used widely in β -N-acetylhexosaminidase-
639 catalyzed transglycosylation reactions.¹⁷⁰⁻¹⁷³ In addition, a wide variety of other synthetic donors
640 such as 4-deoxy-substrates,¹⁷⁴ 1,2-oxazoline containing substrates^{169, 175} and glycosyl azides¹⁷⁶
641 have been developed by researchers to improve such reactions via enhancing yields, substrate
642 specificity and regioselectivity. Nonetheless, the groups released from artificial substrates after
643 transferring GlcNAc or GalNAc moieties to an acceptor are often toxic and not suitable for food
644 applications.¹⁶³ The use of N,N'-diacetylchitobiose ((GlcNAc)₂) has been investigated in some
645 studies for transglycosylation of lactose catalyzed by β -N-acetylhexosaminidases,^{8, 177-178} as it can
646 be obtained as a naturally available, non-toxic and potentially cheaper substrate from chitin
647 depolymerization.¹⁷⁹ In a study by Murata et al.¹⁸⁰ β -N-acetyl-D-hexosaminidase from *Nocardia*

648 *orientalis* was used to transfer *N*-acetylglucosaminyl from (GlcNAc)₂ to *p*NP- β -LacNAc to
649 produce 3'-*O*- and 6'-*O*-*N*-acetylglucosaminyl-*N*-acetyllactosaminide glycosides (GlcNAc-
650 LacNAc-*p*NP), which are believed to carry blood group ABH, Lewis and Ii antigens and act as
651 structural units of polylactosamino glycans. The overall yield and the enzyme regioselectivity were
652 varied by the addition of α -cyclodextrin. This overall yield was relatively low, however, varying
653 between 2.9% to 7.4% using the acceptor *p*NP- β -LacNAc.

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

667 **(Figure 10)**

668

669 ENZYME IMMOBILIZATION

670 The immobilization of enzymes can enhance stability, particularly towards conditions of
671 extreme pH and elevated temperature. The bonds formed between enzymes and the support or
672 carrier potentially reduce the conformational flexibility of the enzyme, thereby increasing
673 resistance to thermal denaturation.¹⁸⁴ Further, it has been demonstrated that higher temperatures
674 can increase the yield of oligosaccharide production¹⁸⁵ and this is possible with a more thermally
675 stable immobilized enzyme.

676 Shiyan et al.¹⁸⁶ immobilized a trans-sialidase from *Trypanosoma cruzi* on ConA-Sepharose
677 for α 2-3 sialylation of the lactosamine type *N*-glycans. This immobilized enzyme was stored at 4
678 °C in the presence of 0.02% NaN₃ for several months without loss of activity and was successfully
679 used for sialylation of oligosaccharides for five cycles.

680 Silica is an attractive support for enzyme immobilization due to its non-toxicity, microbial
681 resistance, ease of handling and robustness towards high flow rates in continuous processes.¹⁸⁷⁻¹⁸⁸
682 Engineered enzymes from *Trypanosoma rangeli* have been immobilized on two silica supports:
683 glyoxyl-functionalized silica and silica functionalized with (3-aminopropyl)triethoxysilane
684 (APTES), which were used for sialidase-catalyzed trans-sialylation by Zeuner et al.¹⁸⁹ While trans-
685 sialylation activity was reduced after immobilization, the glyoxyl-functionalized silica system
686 enhanced productivity by 50% relative to the free enzyme, as long as the enzyme was recycled
687 more than six times. By contrast, glutaraldehyde-APTES-functionalized silica supports reduced
688 the productivity to less than half that of the free sialidase. The glutaraldehyde-APTES method
689 appears to generate a weaker linkage between sialidase and the support compared to the glyoxyl
690 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. Starting 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 Abbaspourrad¹⁹¹ 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 β 1-4 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 β 1,4 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 Fe₃O₄ 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 trisaccharide²⁰⁴ and
765 globotriose²⁰⁵ 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

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

786 The use of membrane technology for separation of a target carbohydrate rather than a
787 mixture, however, is more challenging and less studied in the literature. Separation of sialylated
788 oligosaccharides, such as sialyllactose, sialylated LacNAc and sialylated LNnT, from
789 disaccharides and monosaccharides by nanofiltration has been investigated.^{53,216} Nordvang et al.²¹⁷
790 used a polyethersulfone nanofiltration membrane (NTR-7450) with a molecular weight cut-off of
791 600–800 Da and a zeta potential of –17 at pH=7 to efficiently separate sialyllactose (MW=633
792 Da) from lactose (MW=342 Da). They achieved a high retention of sialyllactose, close to 100%,
793 as a result of both physical and electrostatic barriers. Both sialyllactose and the membrane were
794 negatively charged at the working pH, which resulted in an electrostatic repulsion. Likewise, it is
795 possible to separate sialyl LacNAc from LacNAc, as the molecular weight of sialyl LacNAc (675
796 Da) is greater than that of LacNAc (383 Da).

797 **PERSPECTIVES**

798 This review has highlighted the biological significance and wide application potential of
799 LacNAc-based bioactives, showing that these bioactive molecules may be an important part of the
800 future bioactive market and output of biotech companies. Despite extensive published research on
801 the production of *N*-acetyllactosamine-containing carbohydrates, however, commercial
802 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.

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

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

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1475 **Figure Captions**

1476 **Figure 1.** Structures of *N*-acetylactosamine (LacNAc), Lacto-*N*-neotetraose (LNnT), lacto-*N*-
1477 triose II (LNT2), 6'sialyl LacNAc, 3'sialyl LacNAc, poly-LacNAc, P1 antigen, 6'sialyl LacNAc-
1478 GLP-1, LacNAc-superparamagnetic iron oxide nanoparticles (SPIONs) and 6'sialyl LacNAc-lipo
1479 PGA.

1480 **Figure 2.** Heyns rearrangement of lactulose for synthesis of LacNAc.⁵⁷ Reagents and conditions:
1481 (i) Benzylamine, 40°C, 3 d; (ii) Methanol, glacial acetic acid, 2 h; (iii) HCl, pH 1–2, Pd(OH)₂/C,
1482 H₂, 6 bar, 40°C; (iv) Methanol, Sodium methoxide, Acetic anhydride, 40°C.

1483 **Figure 3.** The linear synthesis of Lacto-*N*-neotetraose (LNnT) (**10**) from a glucosamine
1484 thioglycoside precursor (**1**) and a lactose precursor (**4**).⁶¹ Reagents and conditions: (i) FmocCl, Py,
1485 CH₂Cl₂, 2 h; (ii) HOPO(OBu)₂ NIS/TiOH, CH₂Cl₂, molecular sieves 3 Å, 0 °C, 20 min, 90%; (iii)
1486 TMSOTf, CH₂Cl₂, molecular sieves 3 Å, -30 °C, 15 min, 70%; (iv) 30% Et₃N/CH₂Cl₂, 2 h, 98%;
1487 (v) AgOTf, CH₂Cl₂, molecular sieves 3 Å, -30 °C, 15 min, 84%; (vi) NH₂.NH₂.H₂O, Methanol, 80
1488 °C, 24 h followed by Ac₂O, Methanol, 16 h, 87%; (vii) Pd/C, H₂, EtOH, 16 h, 92%. FmocCl:
1489 fluorenylmethyloxycarbonyl chloride, Et₃N: Triethylamine; NIS: N-iodosuccinimide; TfOH:
1490 triflic acid; TMSOTf: trimethylsilyl trifluoromethanesulfonate; AgOTf: silver
1491 trifluoromethanesulfonate; Ac₂O: acetic anhydride.

1492 **Figure 4.** (A) Coupled microbial synthesis of LacNAc via the combination of two recombinant
1493 *Escherichia coli* strains (*E. coli* NM522/pGT8 and *E. coli* NM522/pNT2/pNT32) and
1494 *Corynebacterium ammoniagenes* DN510. *E. coli* NM522/pNT2/pNT32 and *C. ammoniagenes*
1495 DN510 are attributed to the UDP production system. *E. coli* NM522/pGT8 expressing a β1-4
1496 galactosyltransferase gene of *Neisseria gonorrhoeae* produces LacNAc from UDP-Gal. *ppa*:

1497 pyrophosphatase, *galK*: galactokinase, *lgtB*: β 1-4 galactosyltransferase gene of *N. gonorrhoeae*
1498 F62.⁸¹ (B) Synthesis of lacto-*N*-triose II (LNT2) from lactose using a genetically modified *E. coli*
1499 strain expressing the *N. meningitidis* β 1-3 *N*-acetylglucosaminyltransferase *lgtA* gene. (C)
1500 Synthesis of Lacto-*N*-neotetraose (LNnT) from lactose by co-expression of *N. meningitidis* β 1-4
1501 galactosyltransferase *lgtB* gene with *lgtA*. Lactose transported to the *E. coli* cells by β -galactoside
1502 permease LacY is not hydrolyzed to glucose and galactose because of the lack of the LacZ gene.
1503 LNT2 is released to the extracellular medium while LNnT remains intracellular.⁷⁸

1504 **Figure 5.** Core LacNAc-containing oligosaccharides that can be obtained from lactose using
1505 glycosyltransferases. LacNAc moieties are highlighted in green. iGnT: β 1-3 *N*-
1506 acetylglucosaminyltransferase, IGnT: β 1,6 *N*-acetylglucosaminyltransferase, β 3GalT: β 1-3
1507 galactosyltransferase, β 4GalT: β 1-4 galactosyltransferase, LNT: Lacto-*N*-tetraose, LNnT: Lacto-
1508 *N*-neotetraose, *pLNH*: *para*-Lacto-*N*-hexaose, *pLNnH*: *para*-Lacto-*N*-neo-hexaose, *pLNO*: *para*-
1509 Lacto-*N*-octaose, LNnH: Lacto-*N*-neo-hexaose, LNH: Lacto-*N*-hexaose, *iLNO*: *iso*-Lacto-*N*-
1510 octaose, LND: Lacto-*N*-decaose, LNnD: Lacto-*N*-neo-decaose, LNnO: Lacto-*N*-neo-octaose,
1511 LNO: Lacto-*N*-octaose.²¹⁸

1512 **Figure 6.** (A) LacNAc synthesis by *in situ* regeneration of the nucleotide sugar UDP-Glc and
1513 UDP-Gal from sucrose using a sucrose synthase from rice grains, a UDP-Glc 4'-epimerase from
1514 *Saccharomyces cerevisiae* and a β 1-4 galactosyltransferase from human milk. (B) Repetitive-batch
1515 technique for enhancing enzyme productivity.¹⁰⁵

1516 **Figure 7.** Synthesis of 6' sialyl LacNAc from cytidine-5'-monophosphate-5-*N*-acetylneuraminic
1517 acid (CMP-Neu5Ac) as donor and LacNAc as acceptor using an α 2-6 sialyltransferase (EC
1518 2.4.99.1) with *in situ* generation of *N*-acetylneuraminic acid from *N*-acetylmannosamine

1519 (ManNAc) and pyruvate catalyzed by a sialyl aldolase (EC 4.1.3.3) associated with *in situ*
1520 regeneration of CMP-Neu5Ac catalyzed by a CMP-Neu5Ac synthase (EC 2.7.7.43).¹¹⁰

1521 **Figure 8.** Synthesis of LacNAc-ITag from GlcNAc-ITag and UDP-Gal (Pathway 1) or from
1522 GlcNAc-ITag and lactose (Lac) in the presence of UDP (Pathway 2) using a β 1-4
1523 galactosyltransferases (β 4GalT) from *Neisseria meningitidis* serogroup B strain MC58 (NmLgtB-
1524 B). (B) Synthesis of *p*NP- β -LacNAc from *p*NP- β -GlcNAc and lactose in the presence of UDP
1525 using the β 4GalT.⁷³

1526 **Figure 9.** (A) Reaction mechanism and possible pathways included in β -galactosidase-catalyzed
1527 reactions.²¹⁹ Structures of (B) *N*-acetylneuraminic acid (Neu5Ac), (C) Neu5Ac in an α 2-6-linkage
1528 to galactose and (D) Neu5Ac in an α 2-3-linkage to galactose.²²⁰ (E) Reaction mechanism and
1529 pathways included in sialidase-catalyzed reactions.¹²⁹

1530 **Figure 10.** β -*N*-Acetylhexosaminidase-catalyzed transgalactosylation of lactose for the synthesis
1531 of LNT2 with different GlcNAc containing donors; followed by β -galactosidase-catalyzed
1532 transgalactosylation of LNT2 with lactose as donor for the synthesis of LNnT.

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