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Review

β-*N*-Acetylhexosaminidases for Carbohydrate Synthesis via Trans-Glycosylation

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Abstract: β-*N*-acetylhexosaminidases (EC 3.2.1.52) are retaining hydrolases of glycoside hydrolase family 20 (GH20). These enzymes catalyze hydrolysis of terminal, non-reducing *N*-acetylhexosamine residues, notably *N*-acetylglucosamine or *N*-acetylgalactosamine, in *N*-acetyl-β-D-hexosaminides. In nature, bacterial β-*N*-acetylhexosaminidases are mainly involved in cell wall peptidoglycan synthesis, analogously, fungal β-*N*-acetylhexosaminidases act on cell wall chitin. The enzymes work via a distinct substrate-assisted mechanism that utilizes the 2-acetamido group as nucleophile. Curiously, the β-*N*-acetylhexosaminidases possess an inherent trans-glycosylation ability which is potentially useful for biocatalytic synthesis of functional carbohydrates, including biomimetic synthesis of human milk oligosaccharides and other glycan-functionalized compounds. In this review, we summarize the reaction engineering approaches (donor substrate activation, additives, and reaction conditions) that have proven useful for enhancing trans-glycosylation activity of GH20 β-*N*-acetylhexosaminidases. We provide comprehensive overviews of reported synthesis reactions with GH20 enzymes, including tables that list the specific enzyme used, donor and acceptor substrates, reaction conditions, and details of the products and yields obtained. We also describe the active site traits and mutations that appear to favor trans-glycosylation activity of GH20 β-*N*-acetylhexosaminidases. Finally, we discuss novel protein engineering strategies and suggest potential "hotspots" for mutations to promote trans-glycosylation activity in GH20 for efficient synthesis of specific functional carbohydrates and other glyco-engineered products.

Keywords: glycoside hydrolase family 20; GH20; lacto-*N*-biosidase; human milk oligosaccharides; reverse hydrolysis; enzyme engineering; reaction engineering; regioselectivity; *N*-acetylglucosamine; oxazoline

1. Introduction

N-acetylhexosamines are important constituents in several biological and biochemically significant structures. The most abundant representative is the 2-acetamido-2-deoxy derivative of glucose, namely *N*-acetylglucosamine (GlcNAc), which is the main constituent of chitin—the second most abundant biopolymer on earth. Chitin is found in the exoskeletons of arthropods (e.g., shrimp, crabs, and insects) and in fungal cell walls [1,2]. Furthermore, both GlcNAc and its epimer *N*-acetylgalactosamine (GalNAc) are essential constituents of protein glycosylation structures in eukaryotes as they form part of the glycan core structures.

β-*N*-acetylhexosaminidases (EC 3.2.1.52) of glycoside hydrolase family 20 (GH20) catalyze the hydrolytic removal of *N*-acetylhexosamines from the non-reducing end of *N*-acetyl-β-D-hexosaminides and may act on either *N*-acetylglucosides or *N*-acetylgalactosides (including chains of mixed glucosides as long as either GlcNAc or GalNAc is in the non-reducing end) [3].

Until recently, the β-*N*-acetylhexosaminidases were studied mainly due to their importance in the pathogenesis of the hereditary neuro-metabolic disorders Tay–Sachs and Sandhoff diseases, that both result from mutations in hexosaminidases [3,4]. The microbial β-*N*-acetylhexosaminidases are mainly involved in cell wall synthesis and growth, i.e., in bacteria they act on the peptidoglycan, and in fungi on the chitin. The microbial β-*N*-acetylhexosaminidases have lately received attention due to their potential usefulness in new biosynthetic processes via their ability to catalyze reverse hydrolytic and trans-glycosylation reactions. Such reactions are of significance in novel industrial renewable carbon upcycling and biomimetic glycan synthesis processes.

GlcNAc is probably the hexosamine of the biggest industrial interest since it can be isolated from large industrial waste streams, e.g., from shrimp and crab waste (because chitin is a main constituent of the shrimp and crab shells) and either serve as a renewable carbon and nitrogen source for bio-ethanol production [5] or be used as a constituent for higher value functional applications, e.g., in medicine, high-value food ingredients, or cosmetics [6]. Prominent examples of such high value GlcNAc-containing food-ingredient products are the human milk oligosaccharides (HMOs, Figure 1) [7,8]. Apart from the simplest HMOs such as 2′- and 3-fucosyllactose as well as 3′- and 6′-sialyllactose, all HMOs contain at least one GlcNAc moiety [9]. Because of their beneficial effects for infant development, health, and nutrition [10] and the fact that infant formulae based on cow's milk lack HMOs, several companies have recently started specific research for production of HMOs (e.g., Glycom A/S, Denmark; Abbott Laboratories, USA; Jennewein Biotechnologie GmbH, Germany). In 2016, the first infant formula with a biosynthetically produced GlcNAc-containing HMO (Lacto-*N*-neotetraose (LNnT, Figure 1) produced by Glycom A/S) was successfully brought to the market by Nestlé [11].

Other possible GlcNAc-containing products are non-reducing oligosaccharides as found in some antibiotics (e.g., tunicamycin [12] or orthosomycins [13]), chitooligomers, for which antioxidative [14,15] and antiangiogenic effects [16] have been demonstrated, and branched glycans/oligosaccharides (e.g., HMOs) (Figure 1). Alkylated GlcNAc moieties are part of molecules that can be used as glycosurfactants [17,18] or bivalent lectin ligands [19,20], GlcNAc-modified drugs and vitamins have a higher solubility in water [21,22], and GlcNAc-modified amino acids and glyco-engineered proteins are important for optimal bioactivity [23,24].

In summary, the portfolio of GlcNAc-containing interesting molecules is broad and it would therefore also require a large set of chemical methods and catalysts to synthesize all of them. Especially the chemical synthesis of glycan suffers significantly from laborious protection and de-protection steps. As a result, biocatalytic synthesis involving reverse hydrolytic or trans-glycosylation reactions of GH20 β-*N*-acetylhexosaminidases have recently gained increased attention.

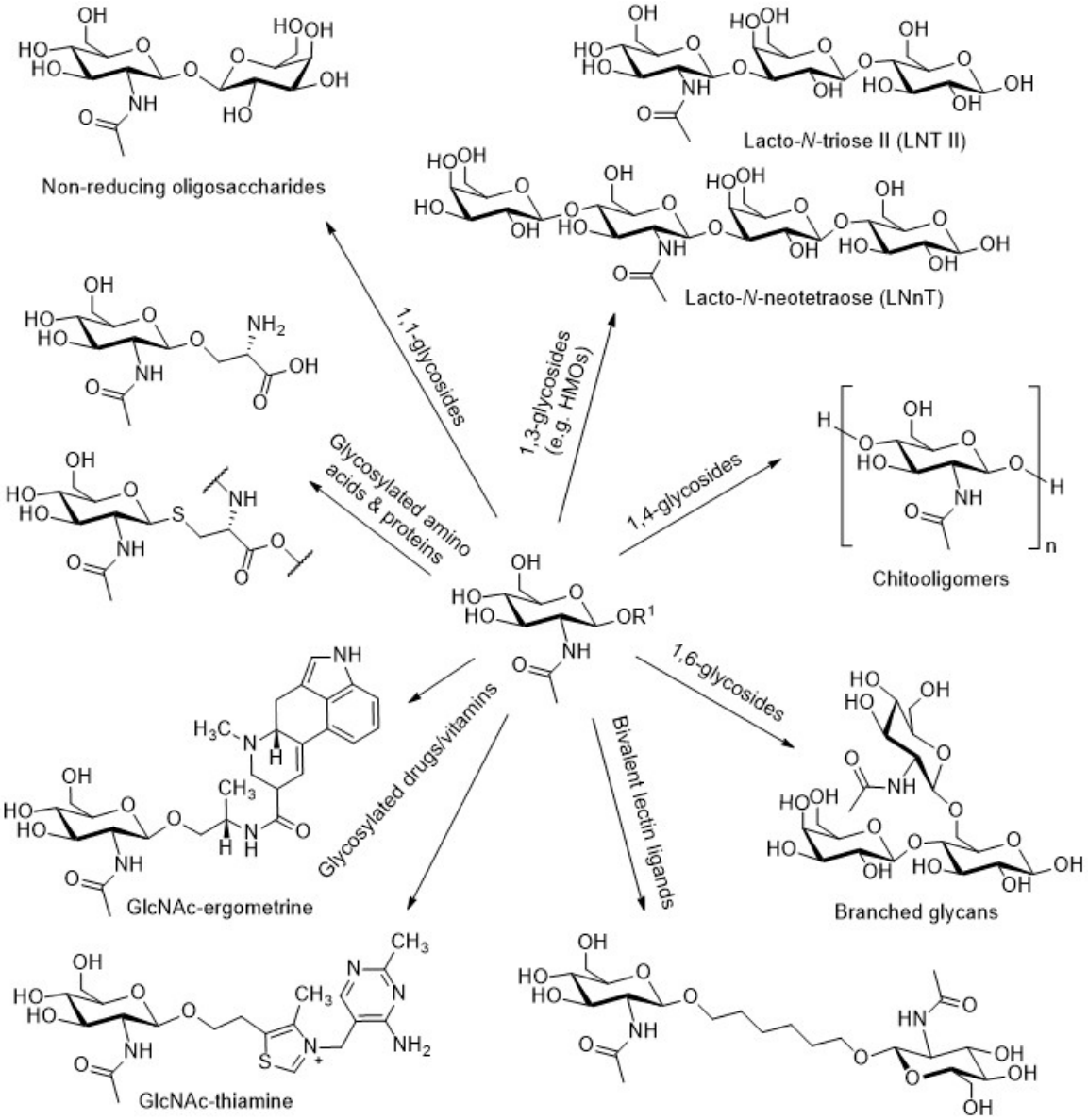

Figure 1. Products from GH20-catalyzed trans-glycosylation reactions.

2. GH20 β-*N*-Acetylhexosaminidases

High-value GlcNAc-containing oligosaccharides or glycosylated molecules can be synthesized using GH20 β-*N*-acetylhexosaminidases. These enzymes can be found throughout the whole tree of life. However, in the CAZy database [25] there are 16 times more bacterial GH20 β-*N*-acetylhexos-aminidase sequences than from any other domain of life. In contrast, there are significantly more studies using fungal β-*N*-acetyl hexosaminidases than of any other origin, which is due to the eminent contribution to this field by Vladimír Křen and colleagues.

Furthermore, GH20 β-*N*-acetylhexosaminidases are usually well-expressed in a recombinant host (either cytosolic or secreted) or can be easily purified from wild-type cultivations since these enzymes are often secreted by their natural host organism. This facilitates simple purification of the enzymes for biocatalytic purposes, which was applied in >95% of the literature studied for this article.

Mechanistically, enzymes from family GH20 are special. In contrast to most other glycoside hydrolases (GHs), the hydrolytic cleavage of the glycosidic linkage to the GlcNAc residues is catalyzed via a so-called substrate-assisted mechanism (Figure 2) [26–30]. In this case, the reaction intermediate is not bound to the enzyme as is the case for the classical Koshland mechanism [31]. The reactive

intermediate is created with assistance of the substrate's 2-acetamido group, which participates in formation of a glucoxazolinium ion intermediate upon cleavage of the glycosidic bond (Figure 2). Whether the intermediate in the GH20 catalysis is actually the oxazolinium ion as proposed for GH84 *O*-GlcNAcases [32] or the uncharged glucoxazoline (Glc-oxa) as proposed for GH18 chitinases [33] is not fully clarified yet. However, it is tempting to speculate that due to the close resemblance of the catalytic mechanisms and motifs of enzymes from families GH20 and GH84 (adjacent Asp-Glu pair), and their proposed substrate-assisted catalytic mechanism, the formation of a glucoxazolinium ion is likely. Nucleophilic attack of water then leads to release of GlcNAc as a reaction product (hydrolysis, Figure 2: $R^1 \neq H$, $R^2 = H$). During this attack the water molecule is stabilized in the active site by a conserved Tyr residue [34]. However, it is a general feature of many retaining GHs that other nucleophiles such as carbohydrates or alcohols are also accepted, which then leads to a glycosylated product (trans-glycosylation, Figure 2: $R^1 \neq H$, $R^2 \neq H$) [35]. Most of the CAZymes acting on GlcNAc residues follow this mechanism (e.g., chitinases (GH18), *O*-GlcNAcases (GH84), endo-β-*N*-acetylglucosaminidases (GH85)) [3,36–38]. Only hexosaminidases from family GH3 [39] and the recently discovered GH136 lacto-*N*-biosidase LnbX from *Bifidobacterium longum* subsp. *longum* [40] were shown to catalyze cleavage of their substrates utilizing the classical double-displacement mechanism involving an enzyme-coupled intermediate.

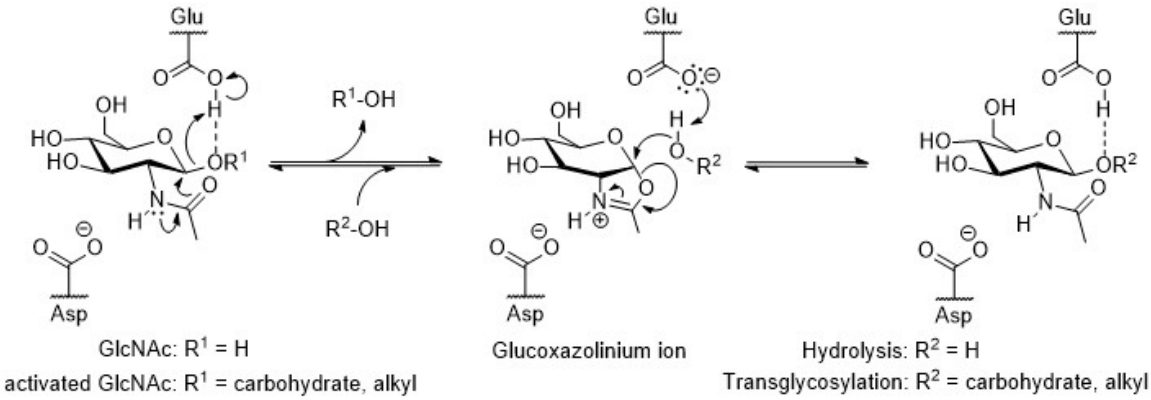

Figure 2. Proposed substrate-assisted mechanism of GH20 β-*N*-acetylhexosaminidases [29]. Indication of the intermediate as a Glc-oxazolinium ion is based on recent computational studies on a GH84 O-GlcNAcase probably utilizing the same mechanism [32].

In nature, glycans, oligo- and polysaccharides as well as other glycosylated molecules are synthesized by glycosyltransferases (GTs), which strictly require a nucleotide-activated derivative as substrate. Therefore, these enzymes are not very attractive for biocatalytic reactions, because the prices for nucleotide-activated sugars are exceptionally high (1,220,000 €/kg (795,000 €/mol) for UDP-GlcNAc at Carbosynth) [41]. Furthermore, functional expression of GTs can be challenging. In contrast, GHs with trans-glycosylase activity are more attractive for large-scale in vitro synthesis of such valuable products. GHs have a broader substrate acceptance and can use non-natural activated carbohydrates or natural disaccharides as donor molecules. However, challenges are also encountered with GH trans-glycosylases. Firstly, the regioselectivity of GH-catalyzed trans-glycosylation reactions can be rather low, which leads to a mixture of the desired product and undesired side products. Secondly, GH trans-glycosylases are in fact hydrolytic enzymes or are derived from such. They are usually still able to hydrolyze both the substrate and the desired product (secondary hydrolysis) [35,42]. However, as outlined in the following, several strategies may be employed to increase trans-glycosylation activity and/or diminish hydrolytic activity in general.

3. Increased Trans-Glycosylation Activity by Reaction Engineering

Since synthesis using GHs can be compromised by secondary hydrolysis and low regioselectivity, a number of reaction engineering strategies, notably including various donor molecule activation approaches, have been reported to help increase yields of GH20-catalyzed trans-glycosylation reactions. Surprisingly, immobilization or continuous product removal approaches, which have been successfully applied for other GH-catalyzed trans-glycosylation reactions [42], have not yet been used to optimize GH20 catalyzed trans-glycosylation reactions.

3.1. Reverse Hydrolysis VS. Trans-Glycosylation

In general, GH-catalyzed synthesis of oligosaccharides or other glycosylated products can be carried out in two different modes of reaction: reverse hydrolysis or trans-glycosylation.

In a reverse hydrolysis reaction, the GH catalyzes a condensation reaction of two carbohydrates (where the activated donor cannot be distinguished among the two reactants) to yield specific disaccharides (Table 1, Figure 2: R^1 = H and $R^2 \neq$ H). The formation of glycosylated amino acids and alkyl glycosides by enzyme-catalyzed reverse hydrolysis have also been described (Table 1). To the best of our knowledge, the term reverse hydrolysis was first used in a review from 1986 [43] and later in the context of glucose-disaccharide formation by β-glucosidase from almond [44], where the authors demonstrated that a high substrate load is required and that a high reaction temperature favors product formation. Ten years later, the first reports on GH20-catalyzed reactions involving the hexosaminidases from *Bacillus circulans* (*Bc*Hex) and *Aspergillus oryzae* (*Ao*Hex) via reverse hydrolysis setup were published [45,46]. Clear disadvantages of such reverse hydrolysis reactions are the long reaction times (72–360 h, Table 1) and the low yields, which rarely exceed 15% isolated yield [23] though higher yields (up to 46%, Table 1) have been reported for non-isolated product outcomes [47].

In contrast, significantly higher yields can be achieved using shorter reaction times in trans-glycosylation reactions (Tables 2–9), where a carbohydrate moiety is transferred from an activated donor molecule to an acceptor molecule containing a free hydroxyl group. The presence of a good leaving group on C1 of the activated donor molecule and a high concentration of acceptor drive the reaction. This approach was used in the first trans-glycosylation reaction reported for a GH20 enzyme by Mega et al. in 1972 [48]. In that pioneering study, the trans-glycosylation ability of the hexosaminidase from *Aspergillus oryzae* (*Ao*Hex) was demonstrated in an auto-condensation reaction of phenyl-activated GlcNAc (Figure 3: GlcNAc-Ph), which mainly led to the formation of GlcNAc-β-1,4-GlcNAc-β-Ph (Table 2). The auto-condensation reaction is a special case of trans-glycosylation, since it involves only one substrate, which acts as both donor and acceptor.

Table 1. Synthetic approaches using GH20 enzymes in reverse hydrolysis reactions. New bonds are highlighted in bold.

Enzyme (Organism)	Acceptor (*c* [mM])	Donor (*c* [mM])	A:D Ratio	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
*An*Hex (*Aspergillus niger* CCIM K2)	GalNAc (1500) [2]		1:1	GalNAc-**α-1,3**-GalNAc (I), GalNAc-**α-1,6**-GalNAc (II)	3.6	35	120	15 (I + II) [3]	[23]
	t-Boc-Ser, *t*-Boc-Thr (1130)	α-GalNAc (1130)	1:1	GalNAc-**α-*O***-Ser (I), GalNAc-**α-*O***-Thr (II)	4.8	35	168	7.4 (I), 3.6 (II) [3,4]	[23]
*Ao*Hex (*Aspergillus oryzae*)	GlcNAc-β-*N*-Ac (1115), GlcNAc-β-*N*-Pr (754)	GlcNAc (113/75)	10:1	GlcNAc-**β-1,6**-GlcNAc-β-*N*-Ac (I), GlcNAc-**β-1,6**-GlcNAc-β-*N*-Pr (II)	5.0	37	120, 168	13 (I), 8 (II) [3,5]	[49]
*Ao*Hex (*Aspergillus oryzae* CCF 1066)	GlcNAc (1500) [2]		1:1	GlcNAc-**β-1,6**-GlcNAc	4.9	39	144	1.9–14.5 [3,6]	[46]
	Alcohols and diols	GlcNAc	n.a.	Alkyl glycosides [7] (**β-*O***)	4.2	37	72	n.a. [6]	[46]
*Ao*Hex (*Aspergillus oryzae* RIB40)	Lac (1218)	GlcNAc (565)	2:1	LNT II (**β-1,3**) (I), GlcNAc-**β-1,6**-Lac (II)	6.0	45	96	0.36 (I), 0.72 (II) [3]	[50]
LnbB (*Bifidobacterium bifidum* JCM1254)	Lac (1000)	LNB (100)	10:1	LNT (**β-1,3**), one unidentified regioisomer [8]	4.5	40	n.a.	n.d. [9]	[51]
*Bc*Hex (*Bacillus circulans*)	Man (9436)	GlcNAc (2260)	4:1	GlcNAc-**β-1,2**-Man (I), GlcNAc-**β-1,6**-Man (II)	5.0	37	360	0.3 (I), 2 (II) [3]	[45]
*Pf*Hex (*Penicillium funiculosus* CCF 1994)	GlcNAc (1031) [2]		1:1	GlcNAc-**β-1,3**-GlcNAc (I), GlcNAc-**β-1,4**-GlcNAc (II), GlcNAc-**β-1,6**-GlcNAc (III)	5.0	37	192	3.8 (I), 1.7 (II), 10 (III) [3,5]	[52]
*Pg*Hex (*Phoma glomerata*)	MeOH (4944)	GlcNAc, GalNAc (90 each)	55:1	GlcNAc-**β-*O***-Me (I), GalNAc-**β-*O***-Me (II)	7.4	37	168	37.8 (I), 46 (II) [9]	[47]

[1] donor based; [2] auto-condensation reaction; [3] isolated yield(s); [4] isolated as de-protected conjugates; [5] isolated as peracetylated derivates; [6] depended on water activity by addition of 0.12–1.17 M $(NH_4)_2SO_4$ or 0.5 M LiCl; [7] product formation only with benzyl alcohol; [8] probably LNB-**β-1,6**-Lac; [9] product(s) not isolated.

Table 2. Auto-condensation reactions of carbohydrate substrates catalyzed by GH20 β-*N*-acetylhexosaminidases. New bonds are highlighted in bold.

Enzyme (Organism)	Substrate (*c* [mM])	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
*Ao*Hex (*A. oryzae*)	pNP-GlcNAc (34)	GlcNAc-**β-1,3**-GlcNAc-β-*O*-pNP	7.0	50	n.a.	5.5 [2]	[53]
[3]	pNP-GlcNAc (24)	GlcNAc-**β-1,4**-GlcNAc-β-*O*-pNP (I), GlcNAc- **β-1,6**-GlcNAc-β-*O*-pNP (II)	6.0	35	6	8 (I), 1.5 (II) [2]	[54]
[3]	$(GlcNAc)_2$ (864)	$(GlcNAc)_3$ (I), $(GlcNAc)_4$ (II) (all **β-1,4**)	6.5	30	25	23.3 (I), 7.5 (II) [2]	[55]
[3]	pNP-GlcNAc (112) [16]	GlcNAc-**β-1,4**-GlcNAc-β-*O*-pNP (I), GlcNAc-**β-1,6**-GlcNAc-β-*O*-pNP (II)	5.5	37	2.5	22.2 (I), 3.8 (II) [2,4]	[56]
[3]	$(GlcNAc)_4$ (161), $(GlcNAc)_3$ (122)	$(GlcNAc)_4$ (I), $(GlcNAc)_5$ (II), $(GlcNAc)_6$ (III) (all **β-1,4**)	6.5	30	54, 52	16 (II) [2,5], 9.2 (III) [2,5]; 13.5 (I) [2,6], 12.7 (II) [2,6], 6.1 (III) [2,6]	[57]

Table 2. *Cont.*

Enzyme (Organism)	Substrate (*c* [mM])	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
[7]	GlcNAc-β-*O*-Ph (38)	GlcNAc-**β-1,4**-GlcNAc-β-*O*-Ph (I), GlcNAc-**β-1,6**-GlcNAc-β-*O*-Ph (II), GlcNAc-**β-1,4**-GlcNAc-**β-1,4**-GlcNAc-β-*O*-Ph (III)	6.0	37	22	2.4 (I), 0.2 (II), 0.2 (III) [2]	[48]
[7]	3-*O*-Me-GlcNAc-β-*O*-Ph, 6-*O*-Me-GlcNAc-β-*O*-Ph, GalNAc-β-*O*-Ph (each 33)	3-*O*-Me-GlcNAc-**β-1,4**-3-*O*-Me-GlcNAc-β-*O*-Ph (I), 3-*O*-Me-GlcNAc-**β-1,6**-3-*O*-Me-GlcNAc-β-*O*-Ph (II), 6-*O*-Me-GlcNAc-**β-1,4**-6-*O*-Me-GlcNAc-β-*O*-Ph (III), GalNAc-**β-1,?**-GalNAc-β-*O*-Ph (IV)	4.5	37	1, 5	12 (I + II), 42 (III), 7.9 (IV) [2]	[48]
*Ao*Hex (*A. oryzae* CCF 1066)	Mixture of GlcNAc and $(GlcNAc)_2$–$(GlcNAc)_7$	Mixture enriched in $(GlcNAc)_2$, $(GlcNAc)_6$, $(GlcNAc)_7$ and $(GlcNAc)_8$ (all **β-1,4**)	4.5	37	9	n.a.	[58]
Hex99 (*Alteromonas* sp. O-7 [20])	$(GlcNAc)_2$ (24)	GlcNAc-**β-1,6**-GlcNAc	7.5	50	0.5	n.a.	[59]
*No*Hex (*N. orientalis* IFO12806 [8])	$(GlcNAc)_2$ (590)	GlcNAc-**β-1,6**-GlcNAc (I), $(GlcNAc)_3$ (**β-1,4**) (II)	5.0	30	40, 6	25 (I), 13 (II) [9]	[60]
*Po*Hex (*P. oxalicum* CCF 2315)	pNP-GlcNPr (46)[17]	GlcNPr-**β-1,4**-GlcNPr-β-*O*-pNP	5.0	37	16	24 [2]	[61]
*Sm*Hex (*S. marcescens* YS-1)	$(GlcNAc)_2$ (94) [18]	$(GlcNAc)_3$ (**β-1,4**)	6.0	40	24	10.7 [9,10], 14.3 [9,11], 26.7 [9,12]	[62]
*Tf*Hex (*Talaromyces flavus* CCF 2686)	pNP-GlcANAc (281)	GlcANAc-**β-1,4**-GlcANAc-β-*O*-pNP	5.0	35	3.5	16 [2]	[63]
	6-SO_3-GlcANAc-β-*O*-pNP (100)	6-SO_3-GlcANAc-**β-1,4**-6-SO_3-GlcANAc-β-*O*-pNP	5.0	35	4	28 [2]	[63]
	pNP-GlcNAc (50)	$(GlcNAc)_2$, $(GlcNAc)_2$-β-*O*-pNP, $(GlcNAc)_3$-β-*O*-pNP (all **β-1,4**)	5.0	35	6	12 [2,13]	[64]
	4-deoxy-GlcNAc-β-*O*-Ph (200)	4-deoxy-GlcNAc-**β-1,6**-4-deoxy-GlcNAc-*O*-Ph	5.0	35	2	14 [2]	[65]
	pNP-GlcNFo (50) [17]	GlcNFo-**β-1,4**-GlcNFo-β-*O*-pNP	5.0	37	3	16 [2]	[61]
	pNP-GlcNGl (50) [19]	GlcNGl-**β-1,4**-GlcNGl-β-*O*-pNP	5.0	37	1.7	78 [2]	[61]
	GlcNAc-β-N_3 (600)	GlcNAc-**β-1,4**-GlcNAc-β-N_3 (I), GlcNAc-**β-1,6**-GlcNAc-β-N_3 (II)	5.0	35	7.5	32 (I), 16 (II) [2]	[66]
a) Y470F	pNP-GlcNAc (50)	$(GlcNAc)_2$-β-*O*-pNP, $(GlcNAc)_3$-β-*O*-pNP, $(GlcNAc)_4$-β-*O*-pNP (all **β-1,4**)	5.0	35	6	41 [2,14]	[64]
b) Y470H	pNP-GlcNAc (50)	$(GlcNAc)_2$-β-*O*-pNP, $(GlcNAc)_3$-β-*O*-pNP, $(GlcNAc)_4$-β-*O*-pNP (all **β-1,4**)	5.0	35	6	26 [2,14]	[64]
c) Y470N	pNP-GlcNAc (50)	$(GlcNAc)_7$-β-*O*-pNP and longer (all **β-1,4**)	5.0	35	6	n.a.[14]	[64]
*Vs*Hex (*Vibrio* sp. P-6-1)	$(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$ (2 each)	Unidentified oligosaccharides (all **β-1,?**) [15]	6.5	40	0.5	n.a.[15]	[67]

[1] donor based; [2] isolated yield(s); [3] purified from β–galactosidase preparation (grade XI, Sigma-Aldrich); [4] isolated from reaction with MeCN; [5] from $(GlcNAc)_4$; [6] from $(GlcNAc)_3$; [7] purified from Taka-diastase (Sankyo); [8] or *Amycolatopsis orientalis* IFO12806T; [9] product(s) not isolated; [10] w/o co-solvent; [11] co-solvent: 1,2,4-butanetriol; [12] co-solvent: 1,3-butanediol; [13] overall yield; [14] no product hydrolysis; [15] only detected by TLC; [16] 491 mM $(NH_4)_2SO_4$, MeCN or dioxane (20% (*v*/*v*) each) added; [17] 45% (*v*/*v*) MeCN added; [18] 8% (*v*/*v*) 1,3-butanediol or 1,2,4-butanetriol added; [19] 5% (*v*/*v*) MeCN added; [20] or *Pseudoalteromonas piscicida*.

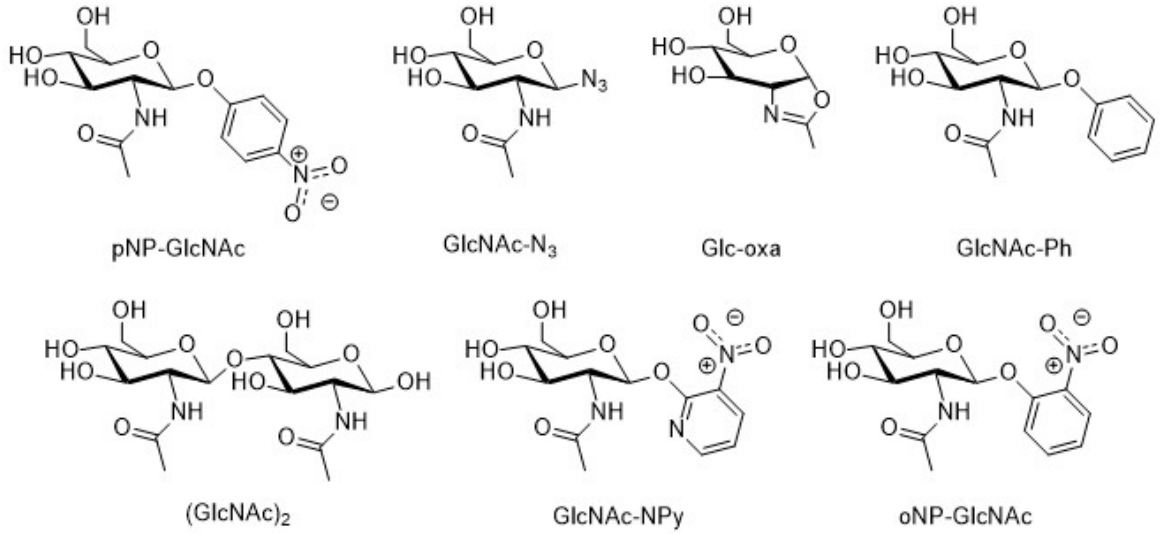

Figure 3. GlcNAc donor substrates used in GH20-catalyzed trans-glycosylation reactions.

3.1.1. *p*-Nitrophenyl Activated Donors

The *p*-nitrophenyl (pNP) derivates of hexosaminides (Figure 3: pNP-GlcNAc) are by far the most popular donor molecules for trans-glycosylation reactions (Tables 3–6). The pNP-glycosides were developed as colorimetric substrates to study hydrolytic GH reactions, because the released pNP can be detected photometrically at λ = 405 nm. However, due to their low price (47,250 €/kg (16,000 €/mol) for pNP-GlcNAc at Carbosynth) [68] and not least the fact that pNP is a good leaving group, they have become attractive as donor molecules for trans-glycosylation reactions. Furthermore, the pNP-hexosaminides are accepted by a wide range of GH20 enzymes as substrate or donor molecule, including fungal (Tables 3 and 4), bacterial (Table 5), and enzymes of other origin (Table 6). Indeed, the first attempt at synthesizing a GlcNAc-containing HMO (lacto-*N*-tetraose (LNT)) utilized a pNP-activated lacto-*N*-biose as donor (Table 5) [69]. However, despite their popularity, the pNP-hexosaminides are not suitable as substrates for synthesis of food products (e.g., HMOs) or in other highly regulated fields, due to the toxicity of the released pNP [70].

3.1.2. Other Activated Donors

In addition to the phenyl-activated donors [48,65,71], other synthetic hexosaminide donor derivatives (Tables 2 and 7) have been used. These have mainly been employed to increase solubility of the donor (e.g., the 2-hydroxy-3-nitro-pyridyl activated GlcNAc (Figure 3: GlcNAc-NPy; Table 7)) [72] or to increase yield and regioselectivity (e.g., the *o*-nitrophenyl (oNP) derivate of α-GlcNAc (Figure 3: oNP-GlcNAc; Table 7)) [73]. Moreover, the glycosyl azide of GlcNAc (Figure 3: GlcNAc-N_3) has been demonstrated to be a superior donor giving higher yields compared to the conventional pNP-GlcNAc, due to its higher solubility (Tables 2 and 7) [66]. However, since the released byproducts from these donor molecules may be irritant (e.g., 2-hydroxy-3-nitro-pyridine) [74], pose an environmental hazard (e.g., oNP) [75], or be acutely toxic (e.g., phenol from Ph-activated [76] and azide from N_3-activated donors [77]), none of these activated donor molecules are feasible for use in synthesis of food components or in similarly regulated applications.

Table 3. Trans-glycosylation reactions catalyzed by GH20 β-*N*-acetylhexosaminidases from *Aspergillus oryzae* using pNP-activated carbohydrate donor substrates. New bonds are highlighted in bold.

Enzyme (Organism)	Acceptor (*c* [mM])	Donor (*c* [mM])	A:D Ratio	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
*Ao*Hex (*A. oryzae*)	6-benzyl-Gal-β-*S*-Et (118, 73)	pNP-GlcNAc (96), pNP-GalNAc (67)	1:1	GlcNAc-**β-1,3**-6-benzyl-Gal-β-*S*-Et (I), GalNAc-**β-1,3**-6-benzyl–Gal-β-*S*-Et (II)	7.0	25	2.5, 30	2.8 (I), 5.7 (II) [2]	[53]
[3]	Glc-α-*O*-pNP (41), GlcNAc-α-*O*-pNP (124)	6-SO_3-GlcNAc-β-*O*-pNP (10/25)	4:1, 5:1	6-SO_3-GlcNAc-**β-1,4**-Glc-α-*O*-pNP (I), 6-SO_3-GlcNAc-**β-1,4**-GlcNAc-α-*O*-pNP (II)	6.0	35	148, 168	35 (I), 94 (II) [2,4]	[54]
[3]	6-SO_3-GlcNAc-β-*O*-pNP (190)	pNP-GlcNAc (30)	6:1	GlcNAc-**β-1,4**-6-SO_3-GlcNAc-β-*O*-pNP	6.0	35	7	13.4 [2]	[54]
[3]	GlcNAc (1157/1112)	pNP-GlcNAc (117/111)	10:1	GlcNAc-**β-1,4**-GlcNAc (I), GlcNAc-**β-1,6**-GlcNAc (II)	6.5	30	40, 215	55 (I) [5], 22 (II) [2]	[55]
[3]	GlcNAc-α-*O*-Me (531), GlcNAc-β-*O*-Me (567)	pNP-GlcNAc (104/97)	5:1, 6:1	GlcNAc-**β-1,4**-GlcNAc-α-*O*-Me (I), GlcNAc-**β-1,4**-GlcNAc-β-*O*-Me (II)	6.5	30	120, 48	51 (I), 24 (II) [2]	[55]
[3]	α-Glc [6], Glc-α-*O*-R; α-GlcNAc, GlcNAc-α-*O*-R (each ca. 1300) [7]	6-SO_3-GlcNAc-β-*O*-pNP (250)	5:1	6-SO_3-GlcNAc-**β-1,4**-Glc-α-*O*-R (I = R^1, II = R^3, III = R^6), 6-SO_3-GlcNAc-**β-1,4**-GlcNAc-α-R (IV = R^5, V = R^1, VI = R^2, VII = R^3, VIII = R^4) [7]	6.0	35	120–144	17 (I), 34 (II), 36 (III), 38 (IV), 51 (V), 87 (VI), 92 (VII), 93 (VIII) [2]	[78]
[3]	Glc-α-*O*-R^3, GlcNAc-α-*O*-R^3, GlcNAc, Glc-α-*O*-R^1, Gal-α-*O*-R^3 (each ca. 1700) [7]	6-SO_3-GlcNAc-β-*O*-pNP (178)	10:1	6-SO_3-GlcNAc-**β-1,4**-Glc-α-*O*-R^3 (I), 6-SO_3-GlcNAc-**β-1,4**-GlcNAc-α-*O*-R (II = R^3, III = R^5, IV = R^1), 6-SO_3-GlcNAc-**β-1,3/6**-Gal-α-*O*-R^3 (V) [7]	6.0	35	74, 66, 370, 71, 48	38 (I), 92 (II), 38 (III), 17 (IV), 25 (V) [2]	[79]
	GlcNAc-β-*N*-Ac (206)	pNP-GlcNAc (21)	10:1	GlcNAc-**β-1,4**-GlcNAc-β-*N*-Ac	5.0	37	4	17 [2,12]	[49]
[3]	Glc-β-*O*-Me, Glc-α-*O*-Me (603 each)	pNP-GalNAc, pNP-GlcNAc (60)	10:1	GalNAc-**β-1,3/4**-Glc-β-*O*-Me (I = **β-1,3**, II = **β-1,4**), GalNAc-**β-1,4/6**-Glc-α-*O*-Me (III = **β-1,4**, IV = **β-1,6**), GlcNAc-**β-1,3/4**-Glc-β-*O*-Me (V = **β-1,3**, VI = **β-1,4**), GlcNAc-**β-1,4/6**-Glc-α-*O*-Me (VII = **β-1,4**, VII = **β-1,6**)	6.5	28	24	49 (I + II), 36 (II I+ IV), 23 (V + VI), 17 (VII + VIII) [2]	[80]
[3]	GlcNAc-β-*O*-Me, GlcNAc-α-*O*-Me (568 each)	pNP-GalNAc (104)	6:1	GalNAc-**β-1,4/6**-GlcNAc-α-*O*-Me (I = **β-1,4**, II = **β-1,6**), GalNAc-**β-1,4**-GlcNAc-β-*O*-Me (III), one unidentified isomer	4.5	30	96	89 (I + II) [2]	[81]
[3]	GlcNAc, GlcNAc-β-*O*-Me, GlcNAc-α-*O*-Me (772 each)	pNP-GlcNAc (76)	10:1	GlcNAc-**β-1,4/6**-GlcNAc (I = **β-1,4**, II = **β-1,6**), GlcNAc-**β-1,4**-GlcNAc-α-*O*-Me (III), GlcNAc-**β-1,4**-GlcNAc-β-*O*-Me (IV), one unidentified isomer	6.5	30	n.a.	55 (I) [8], 22 (II) [8], 55 (III), 24 (IV) [2]	[82]
[3]	Man (3996)	pNP-GlcNAc (38 + 29) [9]	105:1	GlcNAc-**β-1,1/3/4/6**-Man (I = **β-1,1**, II = **β-1,3**, III= **β-1,4**, IV = **β-1,6**)	5.0	37	4	24.6 (I + II + III + IV) [2]	[83]

Table 3. *Cont.*

Enzyme (Organism)	Acceptor (*c* [mM])	Donor (*c* [mM])	A:D Ratio	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
[3]	GalNAc (988/983)	pNP-GlcNAc (140), pNP-GalNAc (141)	7:1	GlcNAc-**β-1,6**-GalNAc (I), GlcNAc-**β-1,4**-GlcNAc (II), GalNAc-**β-1,6**-GalNAc (III)	6.5, 4.5	30	30, 47	26 (I) [5], 38 (III) [2]	[84]
*Ao*Hex (*A. oryzae* CCF 1066)	GlcNAc-β-linker-β-GlcNAc (315, 118)	pNP-GlcNAc (146), pNP-GalNAc (292)	2:1, 1:2	GlcNAc-**β-1,4**-GlcNAc-β-linker-β-GlcNAc (I), GalNAc-**β-1,4**-GlcNAc-β-linker-β-GlcNAc (II)	5.0	35	4.5	7 (I), 6 (II) [2]	[85]
	GlcNAc (230)	pNP-GalNAc (60)	4:1	GalNAc-**β-1,4**-GlcNAc	5.0	37	1	84.5 [2]	[86]
	GlcNAc-α-*O*-R^3 (115), Gal-β-1,4-GlcNAc-α-*O*-R^3 (83) [7]	pNP-GalNAc (29)	4:1, 3:1	GalNAc-**β-1,4**-GlcNAc-α-*O*-R^3 (I), GalNAc-**β-1,6**-GalNAc-β-1,4-GlcNAc-α-*O*-R^3 (II) [7]	5.0	37	4, 1.5	78 (I), 17 (II) [2]	[87]
	GlcNAc-β-1,4-ManNAc (96)	pNP-GalNAc (67)	1:1	GalNAc-**β-1,4**-GlcNAc-β-1,4-ManNAc	5.0	37	2	41 [2,10]	[88]
	Gal (223), GalNAc (197), Lac (189)	pNP-GlcNAc (28/38/24)	8:1, 5:1	GlcNAc-**β-1,1**-Gal (I), GlcNAc-**β-1,4**-GlcNAc-**β-1,1**-Gal (II), GlcNAc-**β-1,6**-GalNAc (III), Lac-**α/β-1,1**-GlcNAc	5.0	37	4.5, 5	17 (I), 7 (II), 14 (III) [2]	[89]
	GlcNAc-β-1,4-ManNAc (104/96)	pNP-GlcNAc (65), pNP-GalNAc (67)	2:1, 1:1	GlcNAc-**β-1,4**-GlcNAc-β-1,4-ManNAc (I), GalNAc-**β-1,4**-GlcNAc-β-1,4-ManNAc (II)	5.0	37	0.8, 2	36 (I), 41 (II) [2,10]	[90]
	pNP-GlcNAc (24)	pNP-GlcNPr (46)	1:2	GlcNPr-**β-1,4**-GlcNPr-β-*O*-pNP (I), GlcNPr-**β-1,4**-GlcNAc-β-*O*-pNP (II)	5.0	37	15.5	4.3 (I), 1.8 (II) [2,11]	[61]

[1] donor based; [2] isolated yield(s); [3] purified from β–galactosidase preparation (grade XI, Sigma-Aldrich); [4] 10% (*v*/*v*) MeCN added; [5] undesired regioisomer digested; [6] no product formation; [7] R^1 = Me, R^2 = Pr, R^3 = allyl, R^4 = pNP, R^5 = OH, R^6 = Ph; [8] first product I formed, later product II; [9] donor added stepwise; [10] 9% (*v*/*v*) MeCN added; [11] 45% (*v*/*v*) MeCN added; [12] isolated as peracetylated derivate(s).

Table 4. Trans-glycosylation reactions catalyzed by fungal GH20 β-*N*-acetyl hexosaminidases using pNP-activated carbohydrate donor substrates. New bonds are highlighted in bold.

Enzyme (Organism)	Acceptor (*c* [mM])	Donor (*c* [mM])	A:D Ratio	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
*Af*Hex (*Aspergillus flavofurcatis* CCF 3061)	Gal (223), GalNAc (197), Lac (189)	pNP-GlcNAc (28/38/24)	8:1, 5:1	GlcNAc-**β-1,1**-Gal (I), GlcNAc-**β-1,4**-GlcNAc-**β-1,1**-Gal (II), GlcNAc-**β-1,6**-GalNAc (III), Lac-**β-1,1**-GlcNAc (IV), Lac-**α-1,1**-GlcNAc (V)	5.0	37	4.5, 5, 7	22 (I) [2], 23.5 (II) [2], 20 (III) [2], 10 (IV) [2,3], 9 (V) [2,3]	[89]
*At*Hex (*Aspergillus tamarii* CCF 1665)	Gal (223), GalNAc (197), Lac (189)	pNP-GlcNAc (28/38/24)	8:1, 5:1	GlcNAc-**β-1,1**-Gal (I), GlcNAc-**β-1,4**-GlcNAc-**β-1,1**-Gal (II), GlcNAc-**β-1,6**-GalNAc (III), Lac-**α/β-1,1**-GlcNAc [4]	5.0	37	4.5, 5	12 (I), 6 (II), 17.5 (III) [2]	[89]
*Pb*Hex (*Penicillium brasilianum* CCF 2155)	6-Ac-GlcNAc (380), GlcNAc (452)	pNP-GlcNAc (42), 6-Ac-GlcNAc-β-*O*-pNP (40)	9:1, 11:1	GlcNAc-**β-1,4**-6-O-Ac-GlcNAc (I), 6-O-Ac-GlcNAc-**β-1,4**-GlcNAc (II)	5.5	37	2, 4.5	16.5 (I), 21 (II) [2,5]	[91]
*Po*Hex (*P. oxalicum*)	GlcNAc (300)	pNP-GlcNAc (30)	10:1	GlcNAc-**β-1,4**-GlcNAc	5.0	35	5.25	13 [2]	[92]
	GalNAc (500)	pNP-GalNAc (30 + 22) [6]	17:1	GalNAc-**β-1,6**-GalNAc	5.0	35	6.25	34 [2]	[92]
*Po*Hex (*P. oxalicum* CCF 2430)	GlcNAc (877), GalNAc (877)	pNP-GalNAc (175/292)	5:1, 3:1	GalNAc-**β-1,4**-GlcNAc (I), GalNAc-**β-1,6**-GlcNAc (II), GalNAc-**β-1,6**-GalNAc (III)	4.5	37	3.5	26.5 (I), 19 (II), 87 (III) [2,7]	[87]
*Tf*Hex (*T. flavus* CCF 2686)	GlcNAc (350)	6-OH-GlcNAc-β-*O*-pNP (50)	7:1	GlcANAc-**β-1,4**-GlcNAc	5.0	37	4	23 [2,8]	[63]
	GlcNAc (300)	6-SO_3-GlcNAc-β-*O*-pNP (75)	4:1	6-SO_3-GlcNAc-**β-1,4**-GlcNAc	5.0	35	6.5	33 [2]	[63]
	GlcNAc (300)	6-OH-GlcNAc-β-*O*-pNP (30)	10:1	GlcANAc-**β-1,4**-GlcNAc	5.0	37	4.5	37 [2,8]	[93]
	GlcNAc-β-1,4-ManNAc (30)	6-OH-GalNAc-β-*O*-pNP (73)	1:2	GalANAc-**β-1,4**-GlcNAc-β-1,4-ManNAc (I), GalANAc-**β-1,4**-GlcNAc (II)	5.0	35	5	35 (I), 39 (II) [2,8]	[88]
*Tf*Hex (*T. flavus* CCF 2686)	GlcNac, GalNAc, Glc, Gal (300)	pNP-GlcNAc, pNP-GalNAc (50)	6:1	GlcNAc-**β-1,4**-GlcNAc (I), GalNAc-**β-1,6**-GalNAc (II), GlcNAc-**β-1,1**-Glc (III), GlcNAc-**β-1,1**-Gal (IV)	5.0	35	5	24 (I), 31 (II), 14 (III), 10 (IV)	[94]
a) Y470F	GlcNAc-β-linker (150)	pNP-GalNAc (50 + 50) [6]	3:1	GalNAc-**β-1,4**-GlcNAc-β-linker	5.0	35	8.5	51 [2,9]	[95]

Table 4. *Cont.*

Enzyme (Organism)	Acceptor (*c* [mM])	Donor (*c* [mM])	A:D Ratio	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
b) Y470H	GlcNAc-β-N_3 (200)	pNP-GalNAc (50)	4:1	GalNAc-**β-1,4**-GlcNAc-β-N_3, GlcNAc-**β-1,4**-GlcNAc-β-N_3	5.0	45	6.5	n.a. [4,10]	[96]
	GlcNAc (200), GlcNAc-β-*O*-EtN_3 (150), GlcNAc-β-N_3 (200)	pNP-GalNAc (50)	4:1, 3:1	GalNAc-**β-1,4**-GlcNAc (I), GalNAc-**β-1,4**-GlcNAc-β-*O*-EtN_3 (II), GalNAc-**β-1,4**-GlcNAc-β-N_3 (III)	5.0	45	4	58 (I), 48 (II), 35 (III) [2,10]	[97]
	MurNAc-β-*O*-Pr (100)	pNP-GalNAc (50)	2:1	GalNAc-**β-1,6**-MurNAc-β-*O*-Pr	5.0	35	5	1	[94]
c) Y470N	GlcNAc-β-linker (50)	pNP-GlcNAc (50 + 25) [6]	1:1	$(GlcNAc)_2$-β-linker (I), $(GlcNAc)_3$-β-linker (II), $(GlcNAc)_4$-β-linker (III) (all **β-1,4)**	5.0	35	8	26.8 (I+II+III) [2]	[64]
	GlcNAc-β-linker (75)	pNP-GlcNAc (50 + 50) [6]	2:1	$(GlcNAc)_2$-β-linker (I), $(GlcNAc)_3$-β-linker (II), $(GlcNAc)_4$-β-linker (III), $(GlcNAc)_5$-β-linker (IV) (all **β-1,4)**	5.0	35	8	58 (I+II+III+IV)	[98]
	GlcNAc-β-*O*-EtN_3 (100)	pNP-GlcNAc (50 + 50) [6]	2:1	$(GlcNAc)_2$-β-*O*-EtN_3 (I), $(GlcNAc)_3$-β-*O*-EtN_3 (II), $(GlcNAc)_4$-β-*O*-EtN_3 (III), $(GlcNAc)_5$-β-*O*-EtN_3 (IV), $(GlcNAc)_6$-β-*O*-EtN_3 (V) (all **β-1,4)**	5.0	35	8	21.5 (I), 18 (II), 11.1 (III), 2.4 (IV), 3.4 (V) [2]	[99]
*Th*Hex (*Trichoderma harzianum* CCF 2687)	UDP-GlcNAc (32)	pNP-GalNAc (208)	1:5	GalNAc-**β-1,4**-GlcNAc-α-UDP	6.5	30	8	22 [2,11]	[100]

[1] donor based; [2] isolated yield(s); [3] isolated as peracetylated derivate(s); [4] yields not determined; [5] 93 mM $(NH_4)_2SO_4$ and 23% (*v*/*v*) MeCN added; [6] donor added stepwise; [7] 20% (*w*/*v*) $MgSO_4$ added; [8] isolated after chemical oxidation of the diol to the carboxylic acid; [9] 40% (*v*/*v*) MeCN added; [10] 10% (v/v) MeCN added; [11] 200 mM β-cyclodextrin added.

Table 5. Trans-glycosylation reactions catalyzed by bacterial GH20 enzymes using pNP-activated carbohydrate donor substrates. New bonds are highlighted in bold.

Enzyme (Organism)	Acceptor (*c* [mM])	Donor (*c* [mM])	A:D Ratio	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
*Au*Lnb (*Aureobacterium* sp. L-101)	Lac (182)	pNP-LNB (18)	10:1	LNT (**β-1,3**)	5.5	40	5	3.7 [2]	[69]
BbhI (*B. bifidum* JCM 1254)	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	55	1.5	46 [3,7]	[101]
	Lac (40)	pNP-GlcNAc (10)	4:1	LNT II (**β-1,3**)	7.0	37	0.6	16 [3]	[102]
	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	55	1.5	45 [2,7]	[103]
	Lac (400)	pNP-GalNAc (10)	40:1	GalNAc-**β-1,3**-Lac	5.8	45	4	55 [2,7]	[103]
a) R577K	Lac (40)	pNP-GlcNAc (10)	4:1	LNT II (**β-1,3**)	7.0	37	0.6	36 [3]	[102]
b) H603F	Lac (40)	pNP-GlcNAc (10)	4:1	LNT II (**β-1,3**)	7.0	37	n.a.	n.a. [3]	[102]
c) D606N	Lac (40)	pNP-GlcNAc (10)	4:1	LNT II (**β-1,3**)	7.0	37	n.a.	9 [3]	[102]
d) D746A	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	55	8	40 [3,4,7]	[101]
	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	37	n.a.	58 [3,7]	[104]
e) D746C/G/S/V	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	37	n.a.	62–73 [3,7]	[104]
f) D746E	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	55	1	71 [3,7]	[101]
	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	37	n.a.	63 [3,7]	[104]
g) D746I	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	37	n.a.	46 [3,7]	[104]
h) D746Q	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	55	6	3 [3,4,7]	[101]
	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	37	n.a.	0	[104]
i) D746T	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	37	3	85 [3,4,7]	[104]
j) D746F/H/K/L/M/N/P/R/W/Y	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	37	n.a.	0	[104]
k) W801H	Lac (40)	pNP-GlcNAc (10)	4:1	LNT II (**β-1,3**)	7.0	37	n.a.	17 [3]	[102]
l) W805A/G/N/S/T/V	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	37	n.a.	45–48 [3,7]	[104]
m) W805C/I/K/L/P	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	37	n.a.	60–69 [3,7]	[104]
n) W805D/E/F/H/M/Q/Y	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	37	n.a.	16–40 [3,7]	[104]
o) W805R	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	37	3	82 [3,7]	[104]
p) Y827F	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	55	1	53 [3,7]	[101]
q) W882H	Lac (40)	pNP-GlcNAc (10)	4:1	LNT II (**β-1,3**)	7.0	37	1.3	66 [3]	[102]
r) D884N	Lac (40)	pNP-GlcNAc (10)	4:1	LNT II (**β-1,3**)	7.0	37	n.a.	12 [3]	[102]
s) RMe4 [10]	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	37	6	75 [3,7]	[104]

Table 5. *Cont.*

Enzyme (Organism)	Acceptor (*c* [mM])	Donor (*c* [mM])	A:D Ratio	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
t) RM34 [11]	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	37	6	69 [3,7]	[104]
u) RMf125 [12]	Lac (400)	pNP-GlcNAc (20)	20:1	LNT II (**β-1,3**)	5.8	37	6	81 [3,7]	[104]
LnbB (*B. bifidum* JCM 1254)	Lac (500)	pNP-LNB (5)	100:1	LNT (**β-1,3**)	4.5	40	n.a.	n.d. [3]	[51]
	Lac (600)	pNP-LNB (20)	30:1	LNT (**β-1,3**)	5.8	37	0.2	8 [3,8]	[105]
a) D320E	Lac (600)	pNP-LNB (20)	30:1	LNT (**β-1,3**)	5.8	37	1	12 [3,8]	[105]
b) D320A	Lac (600)	pNP-LNB (20)	30:1	LNT (**β-1,3**)	5.8	37	27	10 [3,8]	[105]
c) Y419F	Lac (600)	pNP-LNB (20)	30:1	LNT (**β-1,3**)	5.8	37	9	13 [3,8]	[105]
*Sp*HexE314A (*S. plicatus*)	pNP-3/4/6-S-GlcNAc, pNP-3/4-S-GalNAc, pNP-4-S-ManNAc, pNP-4-S-Man (5 each)	pNP-GlcNAc, pNP-GalNAc (30 + 30) [5]	1:6	Different thioglycosides (**β-*S***) [6]	7	37	< 1	>99 [3,9]	[24]

[1] donor based; [2] isolated yield(s); [3] not isolated; [4] no product hydrolysis; [5] donor added stepwise; [6] thioglycoligase reaction; [7] 20% (*v*/*v*) DMSO added; [8] 15% (*v*/*v*) DMSO added; [9] 5% (*v*/*v*) DMSO and 10 mM DTT added; [10] Rme4 = K452M, N500S, I510N, N553S, W805R; [11] RM34 = N500I, M547K, Y594F, N681S, K685R, N702Y, I716A, D746G, S860I; [12] RMf125 = I485F, D746V, I762T, E787A, S889G.

Table 6. Trans-glycosylation reactions catalyzed by mammalian, plant or mollusc GH20 β-*N*-acetylhexosaminidases using pNP-activated carbohydrate donor substrates. New bonds are highlighted in bold.

Enzyme (Organism)	Acceptor (*c* [mM])	Donor (*c* [mM])	A:D Ratio	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
*Bt*Hex (*Bos taurus*)	2-amino-6-benzyl-2-deoxy-Glc-β-*S*-Et (99/112)	pNP-GlcNAc (52), pNP-GalNAc (60)	2:1	GlcNAc-**β-1,3**-2-amino-6-benzyl-2-deoxy-Glc-β-*S*-Et (I), GalNAc-**β-1,3**-2-amino-6-benzyl-2-deoxy-Glc-β-*S*-Et (II)	6.8, 6.3	30	50, 42	3.0 (I), 4.1 (II) [2]	[53]
*Ce*Hex (*Canavalia ensiformis*)	Man-α-*O*-Me (579), Gal-β-*O*-Me (579)	pNP-GlcNAc (62)	9:1	GlcNAc-**β-1,3**-Man-α-*O*-Me (I), GlcNAc-**β-1,3**-Gal-β-*O*-Me (II)	6.5	r.t.	65	16.5 (I), 14.0 (II) [2,3]	[106]
*Cg*Hex (*Chamelea gallina*)	Gal-α-*O*-Me (515), Man-α-*O*-Me (515)	pNP-GlcNAc (50/100)	10:1, 5:1	GlcNAc-**β-1,6**-Man-α-*O*-Me (I), GlcNAc-**β-1,6**-Gal-α-*O*-Me (II)	5.8	r.t.	65	8 (I), 5.6 (II) [2,3]	[73]
	Gal-β-*O*-Me (515/644)	pNP-GlcNAc (50), pNP-GalNAc (73)	10:1, 9:1, 26:1	GlcNAc-**β-1,3**-Gal-β-*O*-Me (I), GlcNAc-**β-1,6**-Gal-β-*O*-Me (II), GalNAc-**β-1,3**-Gal-β-*O*-Me (III)	8.5	37	80, 120	8.8 (I + II), 6.2 (III) [2]	[73]
*Vr*Hex (*Vigna radiata*)	GlcNAc-β-*N*-Ac (138)	pNP-GlcNAc (14)	10:1	GlcNAc-**β-1,6**-GlcNAc-β-*N*-Ac	6.5	37	8	12 [2,4]	[49]

[1] donor based; [2] isolated yield(s); [3] 5% (*v*/*v*) DMF added; [4] isolated as peracetylated derivate(s).

Table 7. Trans-glycosylation reactions catalyzed by GH20 β-*N*-acetylhexosaminidases using other activated carbohydrate donor substrates. New bonds are highlighted in bold.

Enzyme (Organism)	Acceptor (*c* [mM])	Donor (*c* [mM])	A:D Ratio	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
*Ao*Hex (*A. oryzae*) [2]	pNP-Glc (100), pNP-Gal (100)	GlcNAc-β-*O*-NPy (50)	2:1	GlcNAc-**β-1,4**-Glc-β-*O*-pNP (I), GlcNAc-**β-1,4**-Gal-β-*O*-pNP (II)	6.0	25	0.75	8.6 (I), 6.1 (II) [3]	[72]
[4]	Glc, Gal, Man, 2-deoxy-Glc, GlcNAc, GlcNPr, GlcNFo (1100 each), maltose, Lac (570 each)	GlcNAc-β-*O*-Ph (33)	33:1, 17:1	GlcNAc-**β-1,1/2/3/4/6**-Glc (I), GlcNAc-**β-1,?**-Gal (II), GlcNAc-**β-1,?**-Man (III), GlcNAc-**β-1,?**-2-deoxy-Glc (IV), GlcNAc-**β-1,?**-GlcNAc (V), GlcNAc-**β-1,?**-GlcNPr (VI), GlcNAc-**β-1,?**-GlcNFo (VII), GlcNAc-**β-1,?**-maltose (VIII), GlcNAc-**β-1,1**-Lac (IX), autocondensation products	4.5	37	n.a.	36 (I), 38 (II), 3 (III), 10 (IV), 18 (V), 11 (VI), 22 (VII), 24 (VIII), 31 (IX) [5]	[71]
*Cg*Hex (*C. gallina*)	Gal-α-*O*-Me (257)	GalNAc-α-*O*-oNP(10)	26:1	GlcNAc-**α-1,3**-Gal-α-*O*-Me	5.8	37	120	6.3 [3]	[73]
*Tf*Hex (*T. flavus* CCF 2686)	GlcNAc (306)	4-deoxy-GlcNAc-β-*O*-Ph (75)	4:1	4-deoxy-GlcNAc-**β-1,4**-GlcNAc (I), 4-deoxy-GlcNAc-**β-1,6**-GlcNAc (II), 4-deoxy-GlcNAc-**β-1,6**-4-deoxy-GlcNAc-β-*O*-Ph (III)	5.0	35	5.5	7 (I), 6 (II), 14 (III) [3]	[65]
	GalNAc-β-N_3 (400)	GlcNAc-β-N_3 (100)	4:1	GlcNAc-**β-1,6**-GalNAc-β-N_3	5.0	35	3.5	22 [3]	[66]

[1] donor based; [2] purified from β–galactosidase preparation (grade XI, Sigma-Aldrich); [3] isolated yield(s); [4] purified from Taka-diastase (Sankyo); [5] products not isolated.

3.1.3. *N,N′*-Diacetylchitobiose and Chitooligomers as Donor

Potentially more sustainable alternatives to the synthetic donors include the fully *N*-acetylated chitooligomers ($(GlcNAc)_n$) and specifically *N,N′*-diacetylchitobiose (Figure 3: $(GlcNAc)_2$; Tables 2 and 8). These donor substrates are derived from the natural biopolymer chitin and the byproduct of the enzyme catalyzed trans-glycosylation reaction, GlcNAc, is completely biocompatible. Already 30 years ago, it was shown that the hexosaminidase from *Nocardia orientalis* (*No*Hex; now known as *Amycolatopsis orientalis*) is able to synthesize $(GlcNAc)_3$ and GlcNAc-β-1,6-GlcNAc from $(GlcNAc)_2$ in an auto-condensation reaction (Table 2) [60]. The first example utilizing $(GlcNAc)_{3\text{-}4}$ followed three years later in a study of the hexosaminidase from *Vibrio* sp. (*Vs*Hex, Table 2), although the exact products were not identified [67]. Bacterial as well as fungal enzymes, as shown for *Ao*Hex, can utilize $(GlcNAc)_n$ as donor [55,57]. However, the high price for specific chitooligomers and $(GlcNAc)_2$ (3,150,000 €/kg (1,337,000 €/mol) for $(GlcNAc)_2$ at Carbosynth) [107], i.e., even more expensive than UDP-GlcNAc, currently prevents their use as substrates in any industrial process. An alternative process may involve development of safe, cost-efficient extraction of chitooligomers from shellfish waste as outlined previously [8].

3.1.4. Oxazoline-Derivates as Activated Donors

Recently, the oxazoline derivative of GlcNAc (Figure 3: Glc-oxa) has been demonstrated to work as donor molecule for GH20-catalyzed synthesis of the HMO-precursor molecule lacto-*N*-triose II (LNT II, Figure 1) using a mutant of the enzyme Hex1 [108], which was previously discovered in a metagenomic library [109]. Glc-oxa is the natural intermediate of a GH20 reaction and can easily be synthesized using high temperature and alkali [110]. However, oxazolines are unstable at acidic conditions and less useful, eg, in fungal hexosaminidase reactions having acidic pH optima [64]. Nevertheless, the ability of bacterial hexosaminidases with neutral or alkaline pH optima to utilize Glc-oxa as donor for trans-glycosylation reactions as demonstrated (Table 9) [24,101,105,108,111] together with its comparably good price per mole (1,710,000 €/kg (347,000 €/mol) for Glc-oxa at Carbosynth [112]) may increase the popularity of Glc-oxa as donor molecule for such enzymatic synthesis reactions in the future. The fact that there are no byproducts from trans-glycosylation reactions using Glc-oxa and the option of conducting Glc-oxa synthesis under mild conditions [113,114] with enzymatic trans-glycosylation in a cascade fashion [108] might lead to industrialization of GH20-catalyzed trans-glycosylation with Glc-oxa donors.

3.2. Acceptor:Donor Ratio

Another general strategy to increase trans-glycosylation yields is to increase the acceptor:donor ratio (A:D ratio). This approach has proven successful for many trans-glycosylation reactions involving different enzymes as reviewed in [42]. The rationale of this strategy is the direct dependency of the ratio between trans-glycosylation rate and hydrolysis rate on the acceptor concentration [42]. The method has also been widely applied for GH20-catalyzed trans-glycosylation reactions (Tables 1 and 3–10). For carbohydrate acceptor substrates, the A:D ratio is somewhat limited by substrate solubility, but A:D ratios of up to 100:1 have been reported (Tables 3 and 5) [51,83]. Higher ratios of up to 910:1 have been achieved with non-carbohydrate acceptors like alcohols (Table 10) [71], which are usually highly soluble. However, apart from the auto-condensation reactions, for which the A:D ratio is always considered 1:1 (Table 2), a few examples of GH20 trans-glycosylation reactions using an A:D ratio of 1:1 also exist (Tables 1, 3, 4, 9 and 10) [19,23,24,46,52,53,64,88,90,101,115,116]. Most noteworthy in this context is the recently reported synthesis of LNT II where an isolated yield of 86% (281 g/L) was achieved in a reaction using Lac and Glc-oxa in a ratio of 1:1 at a concentration of 600 mM each (Table 9) [100].

Table 8. Trans-glycosylation reactions catalyzed by GH20 β-*N*-acetylhexosaminidases using chitobiose donor substrates. New bonds are highlighted in bold.

Enzyme (Organism)	Acceptor (*c* [mM])	Donor (*c* [mM])	A:D Ratio	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
Hex1 (metagenomic)	Lac (500)	$(GlcNAc)_2$ (100)	5:1	LNT II (**β-1,3**) (I), other regioisomers (**β-1,?**) (II)	8.0	25	2	2 (I), 0.1 (II) [2]	[109]
	Lac (500)	$(GlcNAc)_2$ (100)	5:1	LNT II (**β-1,3**) (I), other regioisomers (**β-1,?**) (II)	7.0	25	8.5	5.7 (I + II) [2]	[117]
a) GTDDA [8]	Lac (500)	$(GlcNAc)_2$ (100)	5:1	LNT II (**β-1,3**) (I), other regioisomers (**β-1,?**) (II)	7.0	25	24	23.3 (I + II) [2]	[117]
b) GTEPG [8]	Lac (500)	$(GlcNAc)_2$ (100)	5:1	LNT II (**β-1,3**) (I), other regioisomers (**β-1,?**) (II)	7.0	25	47	29.5 (I + II) [2]	[117]
Hex2 (metagenomic)	Lac (500)	$(GlcNAc)_2$ (100)	5:1	LNT II (**β-1,3**) (I), other regioisomers (**β-1,?**) (II)	6.0	25	2	8.3 (I), 9.1 (II) [2]	[109]
*No*Hex (*N. orientalis* IFO12806 [3])	pNP-LacNAc (155)	$(GlcNAc)_2$ (730)	1:5	GlcNAc-LacNAc-β-*O*-pNP (three regiosomers: **β-1,3** and **β-1,6**)	5.0	40	12	2.9–7.4 [4,5]	[118]
	Lac-β-*O*-Me (737)	$(GlcNAc)_2$ (324)	2:1	LNT II-β-*O*-Me (**β-1,3**) (I), GlcNAc-**β-1,6**-Lac-β-*O*-Me (II), Gal-β-1,4-(GlcNAc-**β-1,6**)Glc-β-*O*-Me (III)	5.0	40	20	17 (I + II + III) [4]	[119]
	pNP-Lac (168)	$(GlcNAc)_2$ (317)	1:2	LNT II-β-*O*-pNP (**β-1,3**) (I), GlcNAc-**β-1,6**-Lac-β-*O*-pNP (II), Gal-β-1,4-(GlcNAc-**β-1,6**)Glc-β-*O*-pNP (III)	5.0	40	12	2.4 (I + II + III) [4,6]	[119]
	Gal-β-1,3-GalNAc-α-*O*-pNP (23)	$(GlcNAc)_2$ (239)	1:10	Gal-β-1,3-(GlcNAc-**β-1,6**-)GalNAc-α-*O*-pNP (I), GlcNAc-**β-1,6**-Gal-β-1,3-GalNAc-α-*O*-pNP (II), GlcNAc-**β-1,3**-Gal-β-1,3-GalNAc-α-*O*-pNP (III), Gal-β-1,3-(GlcNAc-**β-1,6**-)GalNAc-β-*O*-pNP (IV), GlcNAc-**β-1,6**-Gal-β-1,3-GalNAc-β-*O*-pNP (V), GlcNAc-**β-1,3**-Gal-β-1,3-GalNAc-β-*O*-pNP (VI),	5.0	40	12	14 (I + II + III), 8 (IV + V + VI) [7]	[120]

[1] donor based; [2] not isolated; [3] or *Amycolatopsis orientalis* IFO12806T; [4] isolated yield(s); [5] depended on addition of 0–755 mM α-cyclodextrin; [6] 172 mM β-cyclodextrin added; [7] addition of α-cyclodextrin influenced regioselectivity; [8] specified loop sequence inserted in between W354 and R355 of Hex1.

Table 9. Trans-glycosylation reactions catalyzed by GH20 enzymes using oxazoline-activated carbohydrate donor substrates. New bonds are highlighted in bold.

Enzyme (Organism)	Acceptor (*c* [mM])	Donor (*c* [mM])	A:D Ratio	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
BbhI (*B. bifidum* JCM1254)	Lac (600)	Glc-oxa (60)	10:1	LNT II (**β-1,3**)	7.5	37	2	58 [2]	[101]
a) D746E	Lac (600)	Glc-oxa (60)	10:1	LNT II (**β-1,3**)	7.5	37	0.5	85 [2]	[101]
	Lac (600)	Glc-oxa (600)	1:1	LNT II (**β-1,3**)	7.5	37	0.5	86 [3]	[101]
b) D746A	Lac (600)	Glc-oxa (60)	10:1	LNT II (**β-1,3**)	7.5	37	5	80 [2,4]	[101]
c) D746Q	Lac (600)	Glc-oxa (60)	10:1	LNT II (**β-1,3**)	7.5	37	8	16 [2,4]	[101]
d) Y827F	Lac (600)	Glc-oxa (60)	10:1	LNT II (**β-1,3**)	7.5	37	2.5	80 [2]	[101]
Hex1GTEPG [9] (metagenomic)	Lac (500)	Glc-oxa (100)	5:1	LNT II (I) (**β-1,3**), three unidentified regioisomers (**β-1,?**) (II)	8.0	25	6.5	>25 (I + II) [2]	[108]
LnbB (*B. bifidum* JCM 1254)	Lac (600)	Gal-β-1,3-Glc-oxa (LNB-oxa) (12)	50:1	LNT (**β-1,3**)	7.5	37	0.1	67 [2]	[105]
a) D320E	Lac (600)	LNB-oxa (12)	50:1	LNT (**β-1,3**)	7.5	37	1	29 [2,4]	[105]
b) D320A	Lac (600)	LNB-oxa (12)	50:1	LNT (**β-1,3**)	7.5	37	21	13 [2,4]	[105]
c) Y419F	Lac (600)	LNB-oxa (12)	50:1	LNT (**β-1,3**)	7.5	37	2.5	37 [2]	[105]
*Ph*Nah20A (*Paraglaciecola hydrolytica* S66^T)	Lac (200)	Glc-oxa (100)	2:1	Lac-β-**1,1-β**-GlcNAc (I), LNT II (**β-1,3**) (II), unidentified regioisomer (**β-1,?**) (III) [7]	8.0	37	2	3.8 (I + II + III) [2]	[111]
*Sp*Hex (*S. plicatus*)	Lac (200)	Glc-oxa (100)	2:1	LNT II (**β-1,3**), unidentified regioisomer (**β-1,?**)	8.0	37	1	n.a. [2,8]	[111]
a) E314A	3/4/6-*S*-GlcNAc-β-*O*-pNP, 3/4-*S*-GalNAc-β-*O*-pNP, 4-*S*-ManNAc-β-*O*-pNP, 4-*S*-Man-β-*O*-pNP (5 each)	Glc-oxa (30)	1:6	Different thioglycosides (**β-*S***) [5,6]	7.0	37	<1	>99 [2]	[24]

[1] donor based; [2] not isolated; [3] isolated yield; [4] no product hydrolysis; [5] thioglycoligase reaction; [6] 5% (*v*/*v*) DMSO and 10 mM DTT added; [7] probably Gal-β-1,4-(GlcNAc-**β-1,2**-)Glc or Gal-β-1,4-(GlcNAc-**β-1,3**-)Glc; [8] only analyzed by TLC; [9] specified loop sequence inserted in between W354 and R355 of Hex1.

Table 10. Synthetic trans-glycosylation reactions using non-carbohydrate acceptors catalyzed by GH20 β-*N*-acetyl hexosaminidases. New bonds are highlighted in bold.

Enzyme (Organism)	Acceptor (*c* [mM])	Donor (*c* [mM])	A:D ratio	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
*An*Hex (*A. niger* CCIM K2)	*t*-Boc-Ser, *t*-Boc-Thr (1130)	α-GalNAc (1130)	1:1	GalNAc-**α-*O***-Ser (I), GalNAc-**α-*O***-Thr (II)	4.8	35	168	7.4 (I), 3.6 (II) [2,3]	[23]
*Ao*Hex (*A. oryzae*) [4]	Elymoclavine (95), Chanoclavine (95)	pNP-GalNAc (44)	2:1	GalNAc-**β-*O***-elymoclavine (I), GalNAc-**β-*O***-chanoclavine(II)	4.5	33	60, 28	15 (I), 4.8 (II) [2,5]	[21]
[4]	Ergometrine (95)	pNP-GalNAc (44)	2:1	GalNAc-**β-*O***-ergometrine	4.5	33	26	12 [2]	[21]
[4]	Elymoclavine (95), Chanoclavine (95)	pNP-GlcNAc (44)	2:1	GlcNAc-**β-*O***-elymoclavine (I), GlcNAc-**β-*O***-chanoclavine (II)	4.5	28	70, 52	13.4 (I), 8.1 (II) [2]	[21]
[4]	Ergometrine/Ergometrinine (95)	pNP-GlcNAc (44)	2:1	GlcNAc-**β-*O***-ergometrine, GlcNAc-**β-*O***-ergometrinine	4.5	28	52	10.4 [2]	[21]
[6]	Sphingosine (1670)	GlcNAc (180), Et-GlcNAc (321), Pr-GlcNAc (683) [7]	1:1	GlcNAc-**β-*O***-sphingosine (I), $(GlcNAc)_2$ (**β-1,4**) (II), $(GlcNAc)_3$ (**β-1,4**) (III)	7.0	40	36	44 (I), 14 (II), 8 (III) [2]	[115, 116]
[4]	1,3,5-tris(hydroxyethyl) cyanuric acid (Thca) (77), 1,4-benzenedimethanol (Bm) (145)	pNP-GlcNAc (58 + 2 × 29) [8]	1:1, 3:1	GlcNAc-**β-*O***-Thca (I), GlcNAc-**β-*O***-Bm (II)	5.0	n.a.	>120	29 (I), 12 (II) [2]	[19]
[9]	Several alcohols, ethylene glycol, glycerol, erythritol, three alditols (910 each)	Ph-GlcNAc (1)	910:1	Alkyl-glycosides (**β-*O***) (I) [10], autocondensation products	4.5	37	0.6	27–86 (I) [11]	[71]
*Ao*Hex (*A. oryzae* CCF 1066)	Thiamine (370)	pNP-GlcNAc (125 + 74 + 52) [8]	3:1	GlcNAc-**β-*O***-thiamine	5.0	37	11	3.5 [2]	[22]
	Pyridoxine (195)	pNP-GlcNAc (93 + 4 × 9) [8]	2:1	GlcNAc-**β-*O***-4a-pyridoxine (I), GlcNAc-**β-*O***-5a-pyridoxine (II)	5.0	24	3	13.5 (I + II) [2]	[121]
BbhI (*B. bifidum* JCM1254)	Different alcohols (20% (*v/v*) each)	LNT (1.3)	n.a.	n.d.	6.0	40	n.a.	n.a. [11]	[51]
*No*Hex (*Nocardia orientalis* IFO12806 [13])	1,6-hexanediol (Hx) (50), triethylene glycol (Doo) (51)	$(GlcNAc)_4$ (100)	1:2	GlcNAc-**β-*O***-Hx (I)/Doo (II)-**O-β**-GlcNAc, GlcNAc-**β-*O***-Hx (III)/Doo (IV), $(GlcNAc)_2$-**β-*O***-Hx (V)/Doo (VI) (**β-1,4**)	6.7	40	45	10.5 (I), 22.6 (III), 1.4 (V); 4.7 (II), 69.0 (IV), 0.75 (VI) [2]	[20]
*Po*Hex (*Penicillium oxalicum*)	Cyclohexanol (Chx) (327), coniferyl alcohol (Con) (200)	pNP-GalNAc (30 + 2 × 15) [8]	11:1, 7:1	GalNAc-**β-*O***-Chx (I), GalNAc-**β-1,6**-I (II), GalNAc-**β-*O***-Con (III), GalNAc-**β-1,6**-III (IV) [14]	5.0	35	7	21 (I), 5 (II), 20 (III), 3 (IV) [2]	[92]

Table 10. *Cont.*

Enzyme (Organism)	Acceptor (*c* [mM])	Donor (*c* [mM])	A:D ratio	Product(s)	pH	*T* [°C]	*t* [h]	Yield [%] [1]	Ref.
	t-butanol	pNP-GalNAc (30)	n.a.	Not identified	5.0	35	24	0.7 [11]	[92]
	Myo-inositol (500)	pNP-GalNAc (30 + 2 × 30) [8,19]	17:1	GalNAc-**β-*O*-***myo***-inositol regioisomers I and II	5.0	35	7	3 (I + II) [2]	[92]
	Pyridine-3-aldoxime	pNP-GalNAc (30) [19]	n.a.	Not identified	5.0	35	24	<1 [11]	[92]
*Po*Hex (*P. oxalicum* IFO 5748)	Several alcohols (22% (*v*/*v*) each)	$(GlcNAc)_3$ (16)	236:1–88:1	GlcNAc-**β-*O*-**alkyl	4.5	37	1.5	n.a. [12]	[122]
	6-Benzyloxyhexan-1-ol (Bhx) (955) (20% (*v*/*v*))	$(GlcNAc)_3$ (16)	60:1	GlcNAc-**β-*O*-**Bhx	4.5	37	1	32 [2]	[122]
*Sm*Hex (*Serratia marcescens* YS-1)	Several alcohols, diols and one triol (8% (*v*/*v*) each)	$(GlcNAc)_2$ (94)	21:1–8:1	$(GlcNAc)_3$ (**β-1,4**) (I), GlcNAc-**β-*O*-**alkyl (II)	6.0	40	24	15.3–65.9 (II), 2.2–26.7 (I) [11,15]	[62]
	Several alditols (8% (*w*/*v*) each)	$(GlcNAc)_2$ (94)	6:1–3:1	$(GlcNAc)_3$ (**β-1,4**) (I), GlcNAc-**β-*O*-**alditol (II) [16]	6.0	40	24	1.62–11.0 (II), 8.18–10.4 (I) [11]	[62]
*Sp*HexE314A (*Streptomyces plicatus*)	2,4-dinitrophenol (Dnp), pNP, phenol (20 each), 2-chlorophenol (Cp) (7)	Glc-oxa (5) [20]	4:1, 1:1	GlcNAc-**β-*O*-**Dnp/pNP/Ph/Cp [17]	8.0	37	<1	>99 [11]	[24]
	Et-Cys	pNP-GlcNAc	n.a.	GlcNAc-**β-*S*-**Cys [18]	n.a.	n.a.	n.a.	n.a.	[24]
	Synuclein model peptides: VVCGV (0.002), AGCIA (0.002)	pNP-GlcNAc (10) [21]	1:5000	GlcNAc-**β-*S*-**peptide conjugates (VVCGV (I), AGCIA (II)) [18]	7.0	37	<1	44 (I), 30 (II) [11]	[24]
	Tau protein (244-441) C301S C322S S400C (0.16)	pNP-GlcNAc (6.5)	1:50	GlcNAc-**β-*S*-**Tau conjugate [18]	7.0	37	3	44 [11]	[24]

[1] donor based; [2] isolated yield(s); [3] isolated as deprotected conjugates; [4] purified from β–galactosidase preparation (grade XI, Sigma-Aldrich); [5] 2% diglycoside observed; [6] enzyme source not specified; [7] carried out in a plasticized glass phase: 10% H_2O, 5% EtOH, 5% *n*-PrOH; [8] donor added stepwise; [9] isolated from Taka-diastase (Sankyo); [10] *t*-butanol gave no product; [11] not isolated; [12] only detected by TLC; [13] or *Amycolatopsis orientalis* IFO12806T; [14] only aliphatic OH of coniferyl alcohol glycosylated; [15] 1,3-butanediol and 1,2,4-butanetriol gave no product, but enhanced chitotriose formation; [16] no product formation with dulcitol; [17] Phenyl- and 2-chlorophenylglycosides were cleaved overnight, the other two were stable; [18] thioglycoligase reaction; [19] 30% (*v*/*v*) MeCN added; [20] 5% (*v*/*v*) DMSO added; [21] 10 mM DTT added.

A few examples of successful reactions with A:D ratios <1 (as low as 1:10) have been reported (Tables 4, 5 and 8) [20,24,61,85,88,100,118–120] using carbohydrates as acceptors. The most extreme A:D ratio of 1:5000 was used in a thioglycoligase reaction carried out with a mutant of the hexosaminidase from *Streptomyces plicatus*, which was able to *S*-glycosylate small peptides and proteins [24]. Such a low A:D ratio could only be achieved because of the different type of reaction.

3.3. pH and Temperature Modifying Trans-Glycosylation Activity

In contrast to other trans-glycosylases [123], there are no specific reports of increased trans-glycosylation activity for GH20s by modifying the pH. However, two cases of independent studies using varying conditions for the same enzyme in similar reactions exist. Three independent studies investigated the auto-condensation of pNP-GlcNAc catalyzed by *Ao*Hex at different pH and temperature conditions [53,54,56], resulting in varying regioselectivity (Table 2). Whereas the reaction at neutral pH (pH 7.0) and slightly elevated temperature (50 °C) led to the β-1,3-linked product only [53], the main product of reactions at slightly acidic pH (pH 5.5–6.0) and moderate temperatures (35–37 °C) was the β-1,4-linked product with the β-1,6-linked dimer as side product [54,56]. The higher trans-glycosylation yields obtained in the study using slightly acidic pH [56] are probably due to the higher substrate concentration and solvent addition.

Four independent studies have reported synthesis of LNT II from pNP-GlcNAc and Lac (Figure 4) using the enzyme BbhI from *Bifidobacterium bifidum* (Table 5) [101–104]. Two of the studies used identical conditions for the reaction (pH 5.8, 55 °C, A:D = 20:1, 20% (*v*/*v*) DMSO) and reported similar yields: Both, Chen et al. [103] and Schmölzer et al. [101] thus reported a yield of approximately 45%. In another study, the synthesis of LNT II by BbhI was carried out at neutral pH (pH 7.0) and 37 °C, with an A:D ratio of 4:1 and without DMSO, leading to a yield of 16% (based on HPLC analysis using an internal standard) after 0.6 h [102]. In the other two studies, the D746A and D746E mutants of BbhI, which have been used for LNT II synthesis at pH 5.8 and at two different temperatures of 37 and 55 °C [101,104]. For the mutant D746A, the reaction at the lower temperature (37 °C) resulted in a higher yield of 58% [104] compared to 40% in the other study, where the reaction has been performed at 55 °C [101]. In the case of D746E the opposite trend was observed. The reaction at 37 °C gave a slightly lower yield of 63% [104] compared to 71% [101]. Overall, the addition of DMSO and a lower pH seem to favor trans-glycosylation activity for this specific reaction.

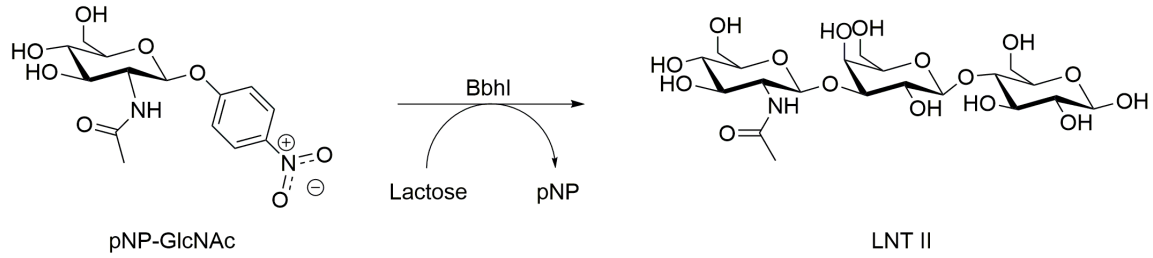

Figure 4. BbhI catalyzed trans-glycosylation reaction for synthesis of LNT II using pNP-GlcNAc as donor substrate.

3.4. Additives Increasing Trans-Glycosylation

Low water activity (a_w) in a solvent system favors synthetic reactions and certain enzymes are known to operate well in such non-conventional systems [124–128]. Addition of co-solvents, water-miscible solutes or salts can alter a_w, leading to an increased synthetic action by hydrolases. However, whereas other hydrolytic enzymes such as proteases, esterases, or lipases can cope with even non-aqueous systems ($a_w < 0.01$), it appears that glycosidases require at least $a_w \geq 0.9$ to be active [129]. Nevertheless, lowering of a_w has been applied for many GHs to increase their trans-glycosylation efficiency [129–136], including GH20 hexosaminidases [21,24,46,54,56,61,62,73,87,88,90–92,95–97,100,101,103–106,115,116,118–120].

3.4.1. Co-Solvents

In general, the most commonly added co-solvent in glycosidase-catalyzed trans-glycosylation reactions is dimethylsulfoxide (DMSO) as it is often required to dissolve the pNP-activated donor substrate. However, there are only a few studies, in which DMSO (5%–20% (*v/v*)) has been present during GH20-catalyzed trans-glycosylation (Tables 5, 9 and 10) [24,101,103–105]. Especially for the fungal enzymes it seems to be more common to add acetonitrile (MeCN, 5%–45% (*v/v*)) as co-solvent to increase yields (Tables 2–4 and Table 10) [54,56,61,88,90–92,95–97]. Other reported co-solvents are *N*,*N*-dimethylformamide (DMF, 5% (*v/v*)) [73,106], dioxane (20% (*v/v*)) [56], 1,3-butanediol and 1,2,4-butanetriol (8% (*v/v*)) [109]. The most extreme case reported in literature is the use of a so-called plasticized glass phase using a total solvent content of only 20% (10% water, 5% ethanol, 5% *n*-propanol) for synthesis of GlcNAc-sphingosine by *Ao*Hex [115,116]. As a consequence of the reaction system, very high substrate concentrations were achieved and an isolated yield of 44% was reported (Table 10).

3.4.2. Salts

Other additives to modulate a_w include inorganic, soluble salts. The concentration-dependent effect of $(NH_4)_2SO_4$ (93–1170 mM) on trans-glycosylation catalyzed by *Ao*Hex and *Pb*Hex (from *Penicillium brasilianum*) resulted in up to seven times higher yields (Tables 1, 2 and 4) [46,56,91]. Addition of LiCl (0.5 M) had similarly positive effects on yields [46]. Analogously, addition of $MgSO_4$ (20% (*w/v*)) led to increased GalNAcase activity and decreased GlcNAcase activity of *Po*Hex (from *Penicillium oxalicum*), which was also applied in trans-glycosylative synthesis of disaccharides [87].

3.4.3. Cyclodextrins

Addition of cyclodextrins is an alternative to co-solvents and salts to improve GH20 trans-glycosylation [100,118–120]. The addition of equimolar amounts of β-cyclodextrin to the acceptor pNP-Lac, which form an inclusion complex shielding the pNP moiety, was reported to not only lead to an increased yield by a factor of two, but also to favor formation of GlcNAc-β-1,6-Gal-β-1,4-Glc over Gal-β-1,4-(GlcNAc-β-1,6)Glc (Table 8) [119]. Similar trends were observed in follow-up studies including use of α-cyclodextrin [118,120]. Later, others have directly compared the use of α-, β-, and γ-hydroxypropyl-β-cyclodextrin as well as heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin for increasing solubility of pNP-GlcNAc and pNP-GalNAc. The latter cyclodextrin had a positive effect on the kinetics for synthesis of GalNAc-β-1,4-GlcNAc-α-O-UDP (increased V_{max} 1.6-fold, increased K_i 5.7-fold) [100].

4. Increased Trans-Glycosylation Activity by Enzyme Engineering

As an alternative to reaction engineering, trans-glycosylation yields can be increased by enzyme engineering. In contrast to the amount of available data for natural GH20 trans-glycosylases, there are only a few studies on engineering these enzymes. Nevertheless, based on the described mutations it is possible to deduce some general mutation guidelines for GH20 β-*N*-acetylhexosaminidase engineering to increase their trans-glycosylation activity. For an overview of hexosaminidase enzyme engineering efforts to improve other protein characteristics (e.g., thermal stability) please refer to the review by Slámová and Bojarová [137].

4.1. Mutation of the Water-Stabilizing Tyr

The first engineering study on a GH20 enzyme was carried out on *Tf*Hex from *Talaromyces flavus* only five years ago [64]. In this work, the authors were inspired by a previous mutational study on a GH85 endo-β-*N*-acetylglucosaminidase, which revealed that mutation of the water stabilizing conserved Tyr residue in the active site to Phe led to increased trans-glycosylation activity and diminished hydrolytic activity [34]. Mutating Y470 in *Tf*Hex (Figure 5A) in a similar manner to Phe led to one of the highest yields (41%) reported for GH20 β-*N*-acetylhexosaminidase catalyzed auto-condensation reactions (Table 2) [64]. Introduction of the hetero-aromatic His residue in this

position also increased the trans-glycosylation yield, though to a somewhat lower degree (Table 2). Interestingly, both mutations also shifted the product spectrum towards longer chito-oligosaccharides ($(GlcNAc)_3$ and $(GlcNAc)_4$). The Y470N mutation, which was inspired by the natural presence of Asn in the same position of closely related GH84 β-*N*-acetylglucosaminidases, even led to synthesis of insoluble oligomers ($(GlcNAc)_7$ and longer, Table 2). However, transferring a similar mutation (Tyr to Phe) to the bacterial enzymes BbhI and LnbB (both from *Bifidobacterium bifidum*) turned out to be less beneficial than in *Tf*Hex (Table 5) [101,105]. In the case of BbhI, the yield of the trans-glycosylation product was increased only to a minor extent when using the pNP-activated donor substrate due to the persistence of secondary hydrolysis. However, when using Glc-oxa as donor at least the synthesis of LNT II by BbhI-Y827F showed a 1.4-fold higher yield (80%) compared to the wild-type (WT) (Table 9) [101]. In contrast, the LnbB-Y419F mutant showed an opposite trend: Whereas the formation of LNT from Lac and LNB-oxa by LnbB-Y419F resulted in a 45% lower yield of the desired product compared to the WT (Table 9), the use of pNP-LNB as donor led to a 1.6-fold higher yield (Table 5) [105]. In summary, mutating the water-stabilizing Tyr to a Phe residue can be a successful strategy to increase trans-glycosylation activity in GH20 enzymes depending on the substrates and enzymes used.

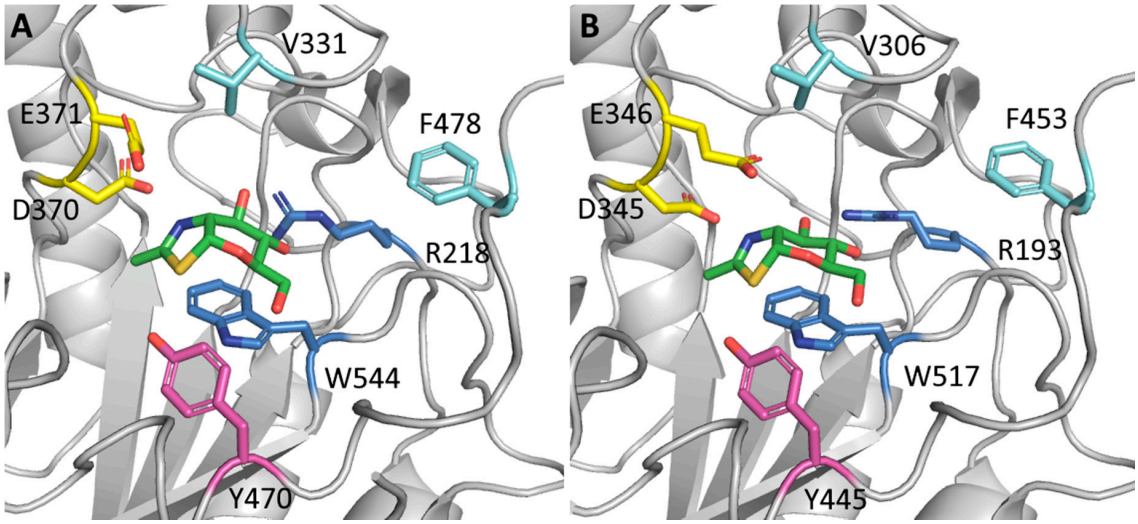

Figure 5. Active sites of two fungal β-*N*-acetylhexosaminidases: (**A**) homology model of *Tf*Hex from *Talaromyces flavus* with the inhibitor Glc-thiazoline docked (created with YASARA [138]); (**B**) crystal structure of *Ao*Hex from *Aspergillus oryzae* (PDB: 5OAR [139]) with the inhibitor Glc-thiazoline bound. The catalytic Asp-Glu pair is highlighted in yellow, the water-stabilizing Tyr residue is highlighted in purple, bound or docked ligands are highlighted in green, conserved residues (Arg and Trp) are highlighted in dark blue, and residues involved in aglycone binding are highlighted in light blue (Phe and Val).

4.2. Mutation of the Aglycone Binding Site

Another possibility to increase trans-glycosylation activity of fungal enzymes was presented at the 13th *Carbohydrate Biotechnology Meeting* in Toulouse, France, in 2019 by researchers from the Křen group: Mutation of the aglycone binding residues V306 and F453 in *Ao*Hex (Figure 5B) to Trp led to an increased trans-glycosylation activity and reduced hydrolytic activity [140]. Due to the close relatedness (78% similarity) the same mutation should be possible in *Tf*Hex (Figure 5A) and other fungal β-*N*-hexosaminidases. However, it seems that this strategy is not applicable to bacterial GH20 enzymes since these have a slightly different aglycone binding site topology, as discussed below.

4.3. Mutation of the Catalytic Asp-Glu Pair

A general and popular strategy to create so called glycosynthases was introduced by Stephen Withers and colleagues over 20 years ago. They demonstrated that a crippled CAZyme, in which

the catalytic nucleophile was mutated to a non-functional Ala residue, is still able to catalyze trans-glycosylation when using a properly activated donor molecule [141]. Due to their relatively simple way of preparation, the glycosyl fluorides became popular as donor molecules for this approach. However, the fluoride approach is not applicable in industrial applications, and was moreover never followed for GH20 enzymes because of the significantly different reaction mechanism. While Glc-oxa should in theory be a properly activated donor molecule, its low stability at low pH hinders the creation of GH20 glycosynthases from fungal β-*N*-acetylhexosaminidases since these require a low pH for optimal activity (Tables 3 and 4). The discovery of bacterial GH20 enzymes with pH optimum ≥7.0 (e.g., Hex1, Table 8) [109] paved the way for the glycosynthase approach in GH20 by mutating the catalytic Asp. The power of such a GH20 glycosynthase for LNT II synthesis (Figure 6) from Lac and Glc-oxa (A:D = 1:1, concentration of both = 600 mM) was recently convincingly demonstrated using the BbhI-D746E mutant, which resulted in 86% isolated yield (281 g/L) in a 30 min reaction at pH 7.5 (Table 9) [101]. This result was a 1.5-fold yield increase compared to the WT (58%; Table 9). Alternative mutations of the same residue (D746A and D746Q) led to real glycosynthases with completely abolished hydrolytic activity, but also to significantly slower enzymes, which was reflected in the lower yield after a longer reaction time (Table 9). Surprisingly, transfer of these mutations to LnbB was not as successful. Both mutants (D320E and D320A, Table 9) were significantly slower in synthesis of LNT from Lac and LNB-oxa (Figure 6) leading to less than 50% of the wild-type yield in more than ten times longer reactions [105]. Finally, a similar 1.5-fold increase in yield compared to the WT was obtained with BbhI-D746E using a pNP-donor molecule, but the obtained yield was much lower (18%; Table 5) [101]. Recently, a new mutational study on BbhI combining directed evolution and site saturation mutagenesis (SSM) showed that the BbhI-D746T led to an almost doubled yield of LNT II when using Lac and pNP-GlcNAc as substrates. The maximum reported yield was 85% (Table 5) [104]. Furthermore, we can conclude from the SSM of position D746 that introduction of any residue bigger than Glu lead to complete inactivation of the enzyme, probably due to steric hindrance of substrate binding, (Table 5). Similarly, introduction of the two amino acid amides Asn and Gln in this position also lead to enzyme inactivation, although varying results were obtained in two independent studies on BbhI-D746Q (Table 5).

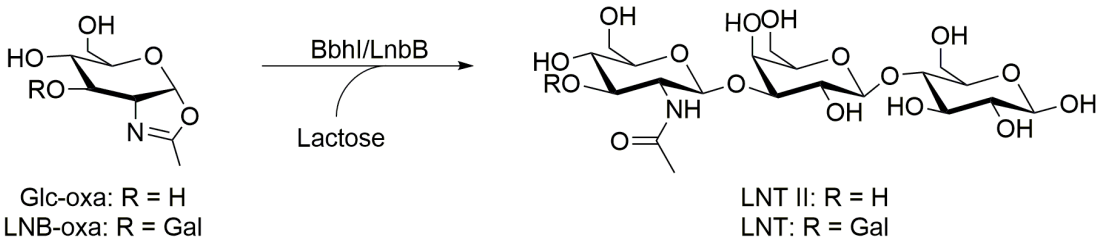

Figure 6. BbhI and LnbB catalyzed trans-glycosylation reaction for synthesis of LNT II and LNT, respectively, using Glc-oxa as donor substrate.

Mutation of the catalytic Glu residue in *Sp*Hex to a non-functional Ala as recently described by Tegl et al. enabled a thioglycoligase reaction (Tables 5, 9 and 10) [24]. Normally, the catalytic Glu residue is required for protonation of the glycosidic bond, which in turn leads to release of the leaving group. Thus, a mutation in this position would be expected to lead to enzyme inactivation. However, the Withers group demonstrated almost 20 years ago that the activity of such a mutated GH can actually be rescued by the use of nucleophiles with a low pK_a value such as thiols, which leads to the formation of thioglycosides [142]. The *Sp*Hex-E314A mutant was not only able to catalyze synthesis of thioglycosides from pNP-GlcNAc and Glc-oxa as donor substrates and the respective thio-sugars as acceptors, but could also catalyze the transfer of a GlcNAc moiety to the free amino acid Cys as well as to Cys-containing peptides and proteins leading to GlcNAc-peptide/protein conjugates [24]. However, such a mutant is not expected to synthesize conventional *O*-glycosidic bonds due to the rather high

pK_a values found in carbohydrates. Product formation of *O*-glycosides was only observed when using the low pK_a nucleophiles pNP and 2,4-dinitrophenol [24].

4.4. Mutation of Other Conserved Active Site Residues

Another generic approach to increase trans-glycosylation activity of CAZymes is the mutation of other conserved residues in the active site to structurally related residues (e.g., from Tyr to Phe), which was first described in 2014 for a GH1 β-glycosidase [143] and has been transferred to many other GH families since then [144–146]. Recently, six conserved positions were identified from an alignment of 585 GH20 sequences and their effect on the trans-glycosylation activity of BbhI was studied [102]. The two mutants R577K and W288H (Figure 7A) led to a doubled and quadrupled yield of LNT II, respectively, in reactions with Lac and pNP-GlcNAc as substrates (Table 5) [102]. These residues are present in both fungal and bacterial GH20 β-*N*-acetylhexosaminidases (residues highlighted in dark blue in Figures 5 and 7). Thus, the corresponding residues could be targeted in other GH20s exhibiting some natural trans-glycosylation activity, as the latter is a requirement for this engineering strategy [102].

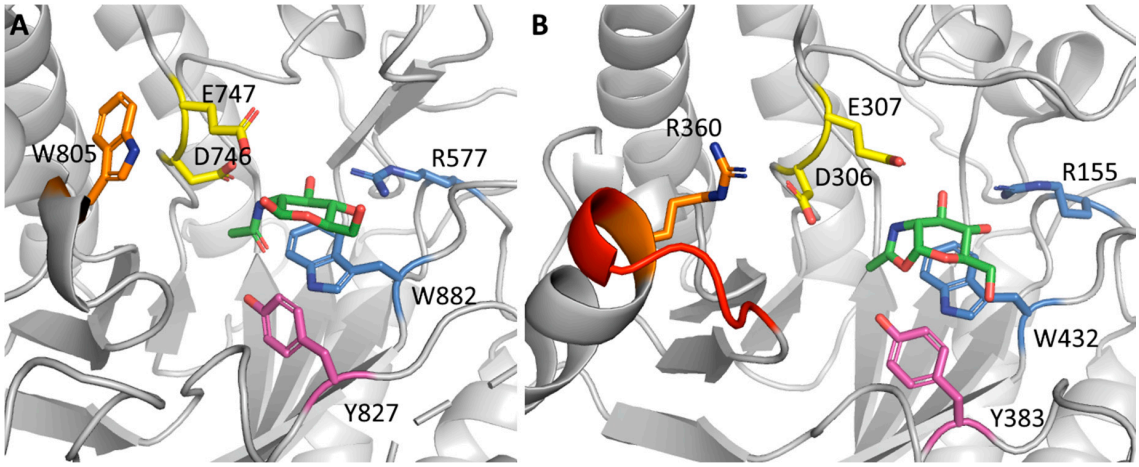

Figure 7. Active sites models of two bacterial β-*N*-acetylhexosaminidases: (**A**) homology model of BbhI from *Bifidobacterium bifidum* (created with YASARA [138]) with the mutated W805 [104] highlighted in orange and GlcNAc bound in the active site; (**B**) homology model of Hex1GTEPG [117] with the inserted loop highlighted in red and the newly positioned R360 in orange; the reaction intermediate Glc-oxa was docked into the structure [117]. The catalytic Asp-Glu pair is highlighted in yellow, the water-stabilizing Tyr residue is highlighted in purple, bound or docked ligands are highlighted in green, and conserved residues (Arg and Trp) are highlighted in dark blue.

4.5. A Non-Conserved Loop Close to the Active Site as Hotspot for Beneficial Mutation?

Lastly, we would like to highlight a potential hotspot for mutations that drive trans-glycosylation activity in GH20 enzymes. We previously demonstrated that introduction of a specific loop, which was identified in related GH20 sequences from pathogens, into Hex1 led to a >5-fold increased trans-glycosylation product yield in reactions with Lac as acceptor and $(GlcNAc)_2$ as donor (Figure 7B, Table 8) [117]. Additionally, in a recent mutational study on BbhI, the mutant W805R (Figure 7A) was identified as the one with superior trans-glycosylation activity [104].

Interestingly, when comparing the homology models of BbhI and the Hex1-GTEPG loop mutant (Figure 7), we noticed that the mutated W805 in BbhI and the newly positioned R360 in Hex1-GTEPG are in a similar position with respect to the active site. Therefore, we dare to speculate that an Arg residue in this position is beneficial for trans-glycosylation reactions using GH20 β-*N*-acetylhexosaminidases. Such an Arg residue might be responsible for modulating the water network or binding water in general, which leads to lowered availability of water for hydrolysis, as proposed for GH33 trans-sialidases [147].

However, in case of Hex1 the inserted loop sequence itself also seems to have an important role with respect to trans-glycosylation activity, since not all alternative loop sequences of the same length were as beneficial as others [117]. Only the additional loops carrying a negative charge in the middle of the five amino acid sequence (GTEPG and GTDDA) led to an increased trans-glycosylation yield. Other tested loops carrying a positive charge (SFRTP) or a negative charge in a different position (DFVTP) led to a decreased trans-glycosylation activity [117].

5. Conclusions

GH20 β-*N*-acetylhexosaminidases are gaining significant attention for production of functional molecules, especially biomimetic human milk oligosaccharides, via their ability to catalyze enzymatic transfer of GlcNAc– and to a lesser extent GalNAc– via reverse hydrolysis or trans-glycosylation. Careful assessment of the available literature data showed that trans-glycosylation is more favorable than reverse hydrolysis with regard to efficiency and product yields. Indeed, a significant body of data have been reported on trans-glycosylation reactions promoted by microbially derived GH20 β-*N*-acetylhexosaminidases. Notably, a large amount of data have been reported for reactions catalyzed by the *Ao*Hex enzyme and the many different mutants of this enzyme derived from *Aspergillus oryzae*, and more recently also for other GH20 enzymes, in particular from *Bifidobacterium bifidum*. Surprisingly, it is not possible to discern any clear trends with respect to pH and temperature on GH20 trans-glycosylation reactions, but reaction engineering involving various types of donor activation, high A:D ratio, high substrate concentration in general, lowering of a_w by addition of co-solvents, salts or addition of cyclodextrins can lead to increased yields or altered regioselectivity. The distinct catalytic mechanism of the GH20 β-*N*-acetylhexosaminidases, involving substrate-assisted catalysis in which the 2-acetamido group acts as an intramolecular nucleophile leading to formation of an oxazolinium ion intermediate, has proven uniquely useful as a blueprint for using oxazoline-conjugated substrates for trans-glycosylation. Although chitin, e.g., from shrimp or crab waste streams, may seem an obvious substrate for sustainable β-*N*-acetylhexosaminidase catalyzed trans-glycosylation processes, we anticipate that oxazoline-conjugated donor substrates have more potential for industrial development of these reactions. Insight into the details of the active site topology and the function of the different amino acids in both the fungal and bacterial GH20 enzymes indicate that trans-glycosylation activity may be promoted by the following protein engineering steps: Mutation of the aglycone binding site to large hydrophobic residues, mutation of the water-stabilizing Tyr as well as other conserved residues, notably Trp and Arg. Efficient glycosynthases using oxazoline substrates can be created by mutation of the catalytic Asp. Recent work suggested that introduction or repositioning of an Arg residue near the active site, e.g., by loop engineering, may be a hotspot for creating beneficial mutations for trans-glycosylation. Based on this foundation, we anticipate the development of novel protein engineering and reaction optimization strategies allowing further technological advances to promote exploration of GH20 enzymes for synthesis of distinct functional carbohydrates and glycan conjugates.

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Abbreviations

For amino acids and nucleotides the standard one and three letter abbreviations were used. For other non-standard abbreviations the reader is referred to the following list:Ac—acetyl, CAZy—Carbohydrate active enzyme, DMF—*N*,*N*-dimethylformamide, DMSO—dimethylsulfoxide, Doo—triethylene glycol, Et—ethyl, EtN_3—azidoethyl, EtOH—ethanol, Gal—galactose, GalNAc—β-*N*-acetylgalactosamine, GH—Glycoside hydrolase, Glc—glucose, GlcANAc—β-*N*-acetylglucurosaminic acid, GlcNAc—β-*N*-acetylglucosamine,

GlcNFo—β-*N*-formylglucosamine, GlcNGl—β-*N*-glycolylglucosamine, GlcNPr—β-*N*-propionylglucosamine, GT—glycosyltransferease, Hx—1,6-hexanediol, Lac—lactose, LacNAc—Gal-β-1,4-GlcNAc, LNB—Lacto-*N*-biose (Gal-β-1,3-GlcNAc), LNT—Lacto-*N*-tetraose (Gal-β-1,3-GlcNAc-β-1,3-Gal-β-1,4-Glc), LNnT—Lacto-*N*-neotetraose (Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc), LNT II—lacto-*N*-triose II (GlcNAcβ-1,3-Gal-β-1,4-Glc), Man—mannose, ManNAc—β-*N*-acetylmannosamine, Me—methyl, MeCN—acetonitrile, N_3—azido, NPy—3-nitro-2-pyridyl, oNP—*ortho*-nitro phenyl, oxa—oxazoline, Ph—phenyl, pNP—*para*-nitro phenyl, Pr—propyl, PrOH—propanol, SSM—site saturation mutagenesis, *t*-Boc—*tert*-butyloxycarbonyl, SO_3—sulfo.

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