

COMMUNICATION TO THE EDITOR

Glycosynthase reaction meets the flow: Continuous synthesis of lacto-*N*-triose II by engineered β -hexosaminidase immobilized on solid support

 Lucija Ruzic¹ | Juan M. Bolivar^{1,2,3} | Bernd Nidetzky^{1,2} 

¹Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Graz, Austria

²Austrian Centre of Industrial Biotechnology, Graz, Austria

³Chemical and Materials Engineering Department, Complutense University of Madrid, Madrid, Spain

Correspondence

Bernd Nidetzky, Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, NAWI Graz, Petersgasse 12, A-8010 Graz, Austria.

Email: bernd.nidetzky@tugraz.at

Abstract

The D746E variant of *Bifidobacterium bifidum* β -*N*-acetyl-hexosaminidase is a promising glycosynthase (engineered glycosidase deficient in hydrolase activity) for the synthesis of lacto-*N*-triose II (LNT II), a core structural unit of human milk oligosaccharides. Here, we develop a flow process for the glycosynthase reaction, which is the regioselective β -1,3-glycosylation of lactose from a *D*-glucosamine 1,2-oxazoline donor. Using the glycosynthase immobilized on agarose beads (~30 mg/g) packed into a fixed bed (1 ml), we show stable continuous production of LNT II (145–200 mM) at quantitative yield from the donor substrate. The wild-type β -*N*-acetyl-hexosaminidase used under exactly comparable conditions gives primarily (~85%) the hydrolysis product *D*-glucosamine. By enabling short residence times (2 min) that are challenging for mixed-vessel types of reactor to establish, the glycosynthase flow reactor succeeds in an effective uncoupling of the LNT II formation (~80–100 mM/min) from the slower side reactions (decomposition of donor substrate, enzymatic hydrolysis of LNT II) to obtain optimum synthetic efficiency. Our study thus provides a strong case for the application of flow chemistry principles to glycosynthase reactions and by that, it reveals the important synergy between enzyme and reaction engineering for biocatalytic synthesis of oligosaccharides.

KEYWORDS

1,2-oxazoline-activated donor substrate, flow chemistry, glycosynthase, human milk oligosaccharides, β -glycosaminidase

1 | INTRODUCTION

Progress in the enzymatic assembly of defined oligosaccharide structures is a core task of applied bio-catalysis in the field of carbohydrates (Danby & Withers, 2016; Mestrom et al., 2019; Nidetzky,

Gutmann, & Zhong, 2018; Zeuner, Jers, Mikkelsen, & Meyer, 2014). Glycosynthases are promising to enable oligosaccharide synthesis with high selectivity and reaction control (Danby & Withers, 2016). Obtained from glycoside hydrolases through mechanistic redesign of their active sites, glycosynthases use a suitably activated donor

Abbreviation: GlcNAc-oxa, *N*-acetyl-*D*-glucosamine 1,2-oxazoline.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2020 The Authors. *Biotechnology and Bioengineering* published by Wiley Periodicals, Inc.

substrate for glycosylation, but are deficient in hydrolysis of the oligosaccharide product formed (Danby & Withers, 2016). Glycosynthase approach to the important class of *N*-acetyl- β -glucosamine-containing oligosaccharides (e.g. lacto-*N*-triose II [LNT II]) entails a specific repurposing of the glycosidase active site, so that the original substrate-assisted catalysis of the enzyme is disrupted, but external 1,2-oxazoline-activated substrate can still be used as donor for glycosylation (Figure 1; Bojarová, Bruthans, & Křen, 2019; Bojarová, Kulik, et al., 2019; Li & Wang, 2018; Slámová & Bojarová, 2017). The D746E glycosynthase of the *Bifidobacterium bifidum* β -*N*-acetyl-hexosaminidase Bbhl catalyzes regioselective β -1,3-glycosylation of lactose from *D*-glucosamine 1,2-oxazoline (GlcNAc-oxa) to give LNT II as the product (Schmölzer, Weingarten, Baldenius, & Nidetzky, 2019; Figure 1a). LNT II is a core structural unit of various biological oligosaccharides including those found in human breast milk. Production of LNT II-based oligosaccharides as nutritional supplements has recently drawn considerable attention (Bode et al., 2016; Chen, 2015; Fajjes, Castejon-Vilatersana, Val-Cid, & Planas, 2019; Zeuner, Teze, Muschiol, & Meyer, 2019).

Besides its excellent activity in the glycosylation of lactose ($\sim 1.3 \times 10^2 \mu\text{mol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$), the D746E glycosynthase shows useful selectivity in the reaction with GlcNAc-oxa (glycosylation/hydrolysis: ~ 7) and combines this with a conveniently low activity for LNT II hydrolysis ($0.06 \mu\text{mol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$; Schmölzer et al., 2019). The glycosynthase thus enables LNT II synthesis (up to $\sim 500 \text{ mM}$; $\leq 1 \text{ hr}$) in high yield ($\sim 86\%$) from donor and acceptor substrates used in equimolar amounts (Schmölzer et al., 2019). Encouraged by the efficiency of the batch reaction, we here developed a flow process for the continuous production of LNT II by immobilized glycosynthase. We show that a packed-bed enzyme reactor facilitates a high-yielding synthesis of LNT II ($\geq 95\%$; $\sim 200 \text{ mM}$) at residence times (2 min) no longer practical for mixed-vessel formats of reactor. The flow process thus boosts the space-time yield ($\sim 1 \mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{min}^{-1}$) by about an order of magnitude compared to the batch conversion. It allows for a complete uncoupling of the fast LNT II formation from slower side reactions, that is, uncatalyzed hydrolysis of the donor substrate and enzymatic hydrolysis of LNT II, to obtain optimum synthetic efficiency. It also integrates enzyme recycling for enhanced total

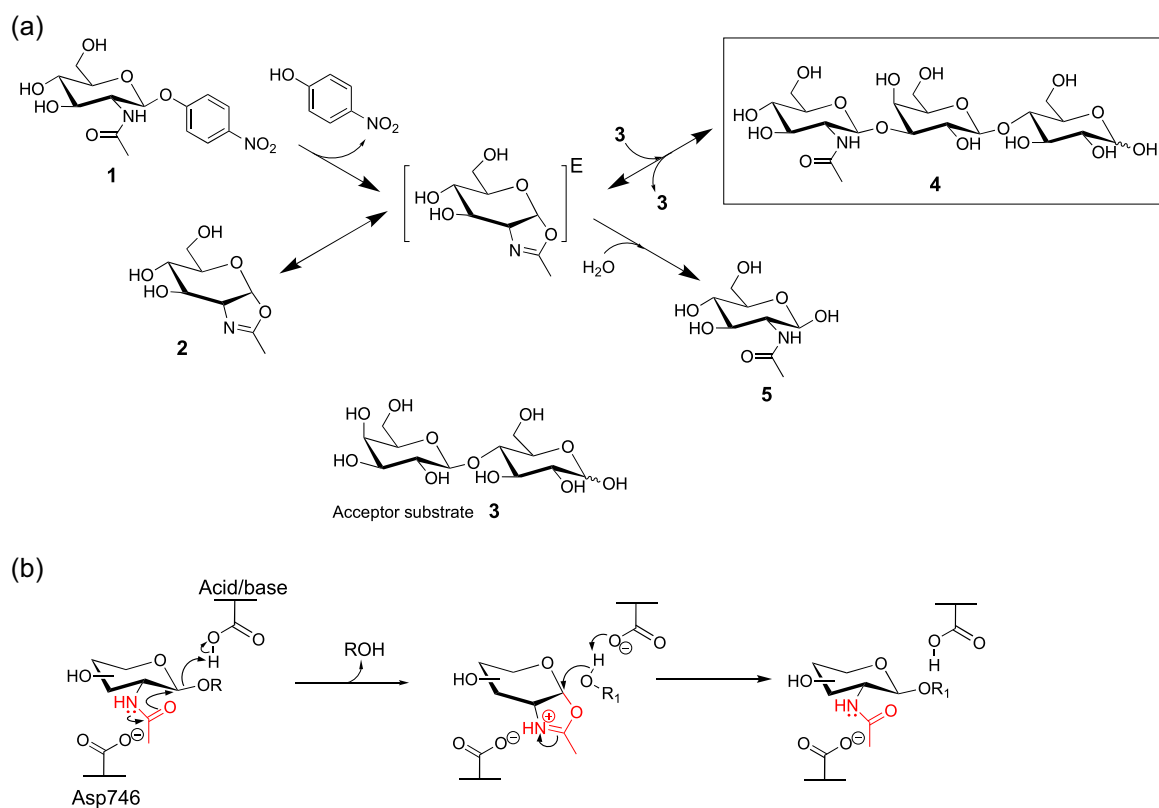


FIGURE 1 Enzymatic reaction for synthesis of lacto-*N*-triose II (LNT II) (4) from *N*-acetyl- β -*D*-glucosamine 1,2-oxazoline (GlcNAc-oxa) (2) and lactose (3) (panel a) and mechanistic basis of the D746E glycosynthase (panel b). As shown in panel a, for assaying the enzyme activity, reaction with 4-nitrophenyl β -*D*-*N*-acetyl glucosaminide (GlcNAc- β -pNP; 1) was used. The released 4-nitro-phenol was measured. Hydrolysis of the enzyme-bound GlcNAc-oxa (2), shown in brackets and indicated with superscript E, yields GlcNAc (5). Note that GlcNAc (5) can be formed directly from the substrate (2) or from the LNT II product (4) via secondary hydrolysis. As shown in panel (b), Asp746 of Bbhl facilitates the formation of the oxazoline intermediate in the enzymatic reaction. Substitution of Asp746 by a glutamic acid elongates the residue side chain by one methylene group and thus interferes with precise positioning of the active-site group for catalysis. Due to destabilization of the formation of the enzyme-bound GlcNAc-oxa intermediate from GlcNAc- β -pNP or LNT II, the D746E glycosynthase is ~ 35 -fold less active than wild-type Bbhl in using these substrates [Color figure can be viewed at wileyonlinelibrary.com]

turnover number of the glycosynthase. In applying flow chemistry principles (Bolivar, Wiesbauer, & Nidetzky, 2011; Tamborini, Fernandes, Paradisi, & Molinari, 2018) to a glycosynthase reaction, we here show the important synergy between enzyme and reaction engineering for biocatalytic synthesis of oligosaccharides.

Wild-type Bbhl and D746E glycosynthase, both produced in *Escherichia coli* and harboring a C-terminally placed hexa-histidine tag (Schmölzer et al., 2019), were immobilized on iminodiacetic acid-activated carriers containing chelated metal ions as surface groups. An initial screening was performed using BCL agarose (4% or 6% crosslinking) and polymethacrylate beads (ReliZyme EP113/S, Purolite ECR8204F), each ligated with Cu^{2+} , Ni^{2+} , or Fe^{3+} (Table S1). Using standard incubations (10 mg protein offered/g carrier; pH 7.5; 150 mM NaCl, 15 mM imidazole; 1.5 hr), we evaluated the immobilization yield (% of the offered activity bound to the carrier) and the effectiveness of the immobilized enzyme (% of the bound activity shown to be active in direct assay). For convenient assay of the enzyme activity, reaction with 4-nitrophenyl β -D-N-acetyl glucosaminide was used (Figure 1a). From the results (Tables S2 and S3), we selected Cu^{2+} -agarose (4%) as the best practically usable option. Both enzymes were immobilized with excellent selectivity, directly from the bacterial cell extract (Table S3; Figure S1). The immobilization yield was useful ($\geq 50\%$) and high enzyme loadings of up to 30 mg protein/g carrier could be obtained in repeated loadings of the carrier. Enzyme binding to Cu^{2+} -agarose was stable, with no elution of activity observed in repeated washings of the beads with buffer. The immobilized enzymes showed a specific activity (cf. effectiveness factor of purified enzyme immobilized on carrier; Table S2) comparable within a two-fold range to the enzyme offered in the solution (D746E glycosynthase: $\sim 50\%$; wild-type enzyme: $\sim 80\%$). We noted that when the wild-type Bbhl was immobilized directly from the *E. coli* cell extract (Table S3), the effectiveness factor of the immobilized enzyme was unexpectedly high (up to $\sim 200\%$). Since the effectiveness factor of the purified enzyme is "normal" ($\leq 100\%$; Table S2), the soluble enzyme activity in the cell extract is probably underestimated in our measurements. Considering the focus of this study on flow process development, the effect was not further pursued. It is worth pointing out that, despite the enzymes' considerable size of ~ 182 kDa (1,566 amino acids), the single hexa-histidine peptide was sufficient to promote immobilization on Cu^{2+} -agarose with remarkably high efficiency and selectivity (Table S3; Figure S1).

Packed bed of enzyme-loaded Cu^{2+} -agarose was assembled in column format (total volume, 1.0 cm^3 ; diameter, 0.62 cm; height, 0.83 cm) and used for continuous-flow experiments (37°C , pH 7.5). Substrate solution (density, $997 \text{ m}^3 \cdot \text{kg}^{-1}$; dynamic viscosity, $9 \times 10^{-4} \text{ kg} \cdot \text{m}^{-1} \cdot \text{s}^{-1}$) was supplied at constant flow rate (volumetric, 0.25 or $0.50 \text{ ml} \cdot \text{min}^{-1}$; axial, 0.21 or $0.42 \text{ cm} \cdot \text{min}^{-1}$) to establish a nominal residence time of 2 or 4 min. Flow is laminar ($Re \leq 10^{-2}$) under these conditions. Release of LNT II and GlcNAc was measured at steady state and monitored over about 10 reactor cycles. First, we analyzed continuous reaction of the wild-type enzyme as reference for the glycosynthase reactions. To use conditions comparable to batch reactions performed previously (Schmölzer et al., 2019), we

supplied the lactose acceptor in ~ 4 -fold excess over the GlcNAc-oxa donor (145 mM). The immobilized wild-type enzyme released only small amounts of LNT II ($\sim 15\%$), as shown in Figure 2a. The main product ($\sim 85\%$) was GlcNAc. GlcNAc-oxa was used up completely in the reaction. The conversion was stable over time (Figure 2a). GlcNAc can be obtained from the hydrolysis of the GlcNAc-oxa donor or the hydrolysis of LNT II product. The wild-type enzyme is rather unselective in using GlcNAc-oxa for reaction with water (hydrolysis) or reaction of lactose (glycosylation). However, the LNT II yield in the continuous reaction (Figure 2a) was much lower than expected from completely unselective usage of the GlcNAc-oxa donor ($\sim 50\%$; Schmölzer et al., 2019). The specific activity of wild-type Bbhl for LNT II hydrolysis is $1.9 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (Schmölzer et al., 2019). Considering the protein loading ($\sim 25 \text{ mg} \cdot \text{ml}^{-1}$) in the continuous reaction (Figure 2a) and assuming an effectiveness factor of 0.8 for the immobilized enzyme (Table S2), we estimate the rate of LNT II hydrolysis (upper limit) as $\sim 39 \mu\text{mol} \cdot \text{mL}^{-1} \cdot \text{min}^{-1}$ ($= 1.9 \times 25 \times 0.83$). Although LNT II synthesis is at least ~ 20 -fold faster than hydrolysis (Table 1 in Schmölzer et al., 2019) the fast hydrolysis rate narrows down the operational range of the residence times for product synthesis, such that a continuous production of LNT II is no longer practically possible.

Dramatic improvement in LNT II yield due to replacement of wild-type Bbhl by the D746E glycosynthase is demonstrated in Figure 2b. The GlcNAc-oxa donor was used completely (90–100%) for LNT II production ($\geq 130 \text{ mM}$) and conversion was stable over several reactor cycles. Besides showing 7-fold higher selectivity than wild-type Bbhl in utilizing GlcNAc-oxa for glycosylation as compared to hydrolysis (Table 1 in Schmölzer et al., 2019), the immobilized D746E glycosynthase was estimated to catalyze LNT II hydrolysis at a rate of only $\sim 0.86 \mu\text{mol} \cdot \text{mL}^{-1} \cdot \text{min}^{-1}$ ($= 0.06 \times 30 \text{ (mg} \cdot \text{mL}^{-1}) \times 0.48$ (effectiveness from Table S2)). The corresponding synthesis rate was estimated from earlier data (Figure 3b in Schmölzer et al., 2019) as $\sim 3.9 \times 10^2 \mu\text{mol} \cdot \text{mL}^{-1} \cdot \text{min}^{-1}$ ($= 27 \times 30 \times 0.48$). Thus, the LNT II synthesis/hydrolysis rate ratio was at least ~ 450 . The slow hydrolysis rate resulted in broadening of the operational window for the usable residence times to the min range. This was crucial for the continuous production of LNT II to become practically feasible.

We then studied the continuous reaction of the D746E glycosynthase with donor and acceptor substrate used at similar concentrations. Supplying GlcNAc-oxa in 1.2-fold molar excess over lactose (200 mM), LNT II was released in quantitative yield based on the limiting substrate used (Figure 2c). A LNT II concentration of $109 \text{ mg} \cdot \text{mL}^{-1}$ was obtained in the product stream and the space-time yield was $54.5 \text{ mg} \cdot \text{mL}^{-1} \cdot \text{min}^{-1}$. Based on the amount of enzyme used, a specific productivity of $1.8 \text{ mg LNT II} \cdot \text{mg}^{-1} \text{ immobilized enzyme mL}^{-1} \cdot \text{min}^{-1}$ can be calculated. In the eight reactor cycles performed over 16 min, a total amount of 0.87 g LNT II was synthesized. The mass-based enzyme turnover at this stage is 29 ($=870/30$) mg LNT II/mg protein. Further scale-up of the reaction volume processed could be achieved conveniently, simply by increasing the number of reactor cycles used. The enzyme turnover could be also increased in that way. Furthermore, the size of the fixed-bed enzyme reactor could be also increased easily.

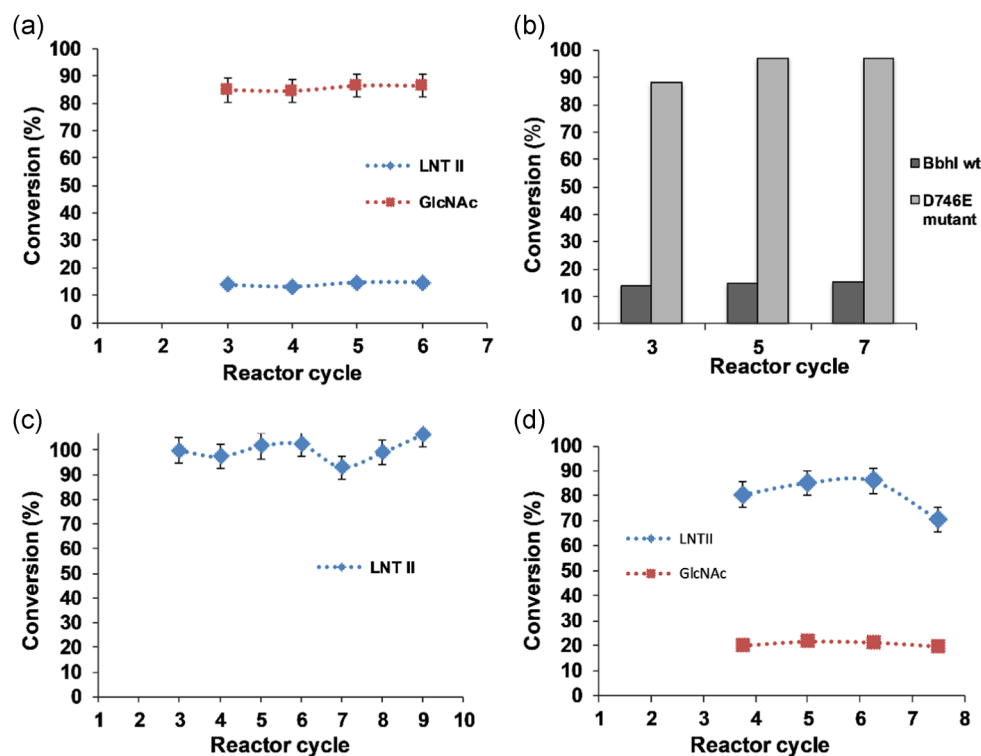


FIGURE 2 Flow synthesis of lacto-*N*-triose II (LNT II). Reactions were performed at 37°C and pH 7.5. The conversion of the *N*-acetyl-D-glucosamine 1,2-oxazoline (GlcNAc-oxa) substrate into the products of synthesis (LNT II) and hydrolysis (GlcNAc) is shown. (a,b) Reactions of immobilized wild-type Bbhl (a) and D746E glycosynthase in comparison to the wild-type enzyme (b). Panel b shows conversion of GlcNAc-oxa into LNT II. Conditions: 145 mM GlcNAc-oxa, 600 mM lactose, 2 min residence time, 25 mg/g (wild-type) and 30 mg/g (D746E; five steps of immobilization). (c,d) Reactions of immobilized D746E glycosynthase at 2 min (c) and 4 min (d) residence time. Conditions 200 mM lactose, 282 mM (c) and 165 mM (d) GlcNAc-oxa [Color figure can be viewed at wileyonlinelibrary.com]

Despite the relatively low hydrolase activity against LNT II, the synthetic reaction of the D746E glycosynthase nonetheless required control of the residence time. We show in Figure 2d that an increase in residence time to 4 min went along with a marked decrease in the LNT II yield to ~83%. The reaction was performed with lactose (200 mM) in 1.2-fold molar excess over GlcNAc-oxa to minimize the effect of hydrolysis of the donor substrate. The GlcNAc released (~17%) thus originated primarily from the progressed hydrolysis of LNT II.

In summary, we demonstrate in this study the efficient biocatalytic production of LNT II from GlcNAc-oxa and lactose using a flow process based on immobilized D746E glycosynthase. Principles of continuous reaction engineering are applied, to our knowledge for the first time, to a glycosynthase-catalyzed glycosylation. We show that the operational range of residence times (≤ 5 min) made accessible specifically through the flow process was essential for an effective synthesis, in which the β -1,3-glycosylation of lactose is decoupled from hydrolysis of the LNT II product. We also show that the use of D746E glycosynthase was crucial. The synthesis could not have been performed similarly with the wild-type Bbhl due to high hydrolase activity of this enzyme. Our study thus suggests the important synergy between enzyme and reaction engineering for biocatalytic synthesis of defined oligosaccharides using glycosynthases.

2 | MATERIALS AND METHODS

2.1 | Materials, substrates, and enzymes

Unless mentioned, the materials and chemicals used were those reported by Schmölzer et al. (2019). GlcNAc-oxa was prepared by chemical synthesis from GlcNAc, as described in Schmölzer et al. (2019). Wild-type Bbhl and its D746E glycosynthase variant were produced as C-terminally His-tagged proteins in *E. coli*. The enzymes were purified by affinity chromatography. The experimental procedures of Schmölzer et al. (2019) were used. BCL agarose (4% or 6% crosslinking) was from Agarose Bead Technologies (Madrid, Spain). ReliZyme EP113/S was gift of Resindion (Binasco, Italy). Purolite ECR8204F was a gift of Purolite GmbH (Ratingen, Germany).

2.2 | Enzyme immobilization

Immobilization carriers were chemically functionalized with iminodiacetic acid groups and loaded with Ni^{2+} , Cu^{2+} , or Fe^{3+} . Procedures from literature (Mateo, Grazu, & Guisan, 2013) were applied and full details are given in the Supporting Information along with a summary of the physical characteristics of the carriers used. Immobilization was performed at 10%

(wt/vol) loading of carrier in 20 mM sodium phosphate buffer, pH 7.5, containing 150 mM NaCl and 15 mM imidazole. When instead of purified enzyme (~10 mg protein·g⁻¹ carrier) the *E. coli* cell extract (40–140 mg total protein·g⁻¹ carrier) was used in the immobilization, the imidazole concentration was raised to 50 mM to reduce nonspecific protein binding. The activity loading in single-step immobilization was 50–160 U·g⁻¹ for wild-type enzyme and about 4–5 U·g⁻¹ for D746E glycosynthase. To enhance the total activity immobilized on the carrier, the immobilization was performed in up to five successive steps, with a buffer washing step in between. Immobilization was done under gentle agitation in an end-over-end rotator at ~20°C for 1.5 hr and was monitored from the decrease in protein and activity in the supernatant. Enzyme activity was also measured directly from the solid carrier. The immobilization yield is the ratio between the enzyme activity removed from the supernatant during immobilization and the offered enzyme activity. The effectiveness factor is the ratio between the enzyme activity measured directly on the solid carrier and the enzyme activity removed from the supernatant.

2.3 | LNT II synthesis in continuous flow

A 1-ml (diameter, 0.62 cm; height, 3.3 cm) Proteus FliQ FPLC Column (Generon, San Prospero, Italy) was packed with 0.958–0.969 g carrier containing immobilized wild-type or D746E enzyme. The packed volume was 1.0 ml (height, 0.83 cm). The inlet and outlet of the column was connected with Teflon tubing (diameter, 250 µm; Micronit Microfluidics; Enschede, The Netherlands). The column was placed in a water bath kept at 37°C. Liquid flow was delivered from a New Era NE-1000 syringe pump (Next Advance, Troy, NY). A 50 mM sodium phosphate buffer, pH 7.5, was used. The concentrations of GlcNAc-oxa and lactose were varied as indicated. Samples were collected at outlet, heat-treated (10 min, 99°C), and analyzed by high-performance liquid chromatography (HPLC). Control incubations were performed in the absence of enzyme. No LNT II was produced over 24 hr. No evidence of formation of another oligosaccharide was obtained. The GlcNAc-oxa was however hydrolyzed gradually. Under incubation at elevated temperature (99°C, 10 min), no LNT II was formed, but the GlcNAc-oxa was hydrolyzed. As shown previously (Schmölzer et al., 2019), LNT II formation is strictly correlated with the enzyme activity.

2.4 | Assays and analytical procedures

Enzyme activity in solution or immobilized on solid carrier was measured at 37°C (20 mM sodium phosphate, pH 7.3; 20 wt% DMSO) using GlcNAc-β-pNP (20 mM) as the substrate. Lactose (400 mM) was added as an acceptor to mimic the conditions of the synthetic trans-glycosylation. The release of 4-nitro-phenol was monitored at 405 nm. The specific activity of the purified D746E glycosynthase and the wild-type enzyme was determined as 0.46 and 16 µmol·mg⁻¹·min⁻¹, respectively. In the *E. coli* cell extract, the corresponding activities were 0.03 and 2.49 µmol·mg⁻¹·min⁻¹. Protein was measured by absorbance at 280 nm (SA-11 + Spectrophotometer; DeNovix Inc., Wilmington, NC). LNT II and

GlcNAc were analyzed by HILIC–HPLC using a Luna NH₂ column (3 µm, 100 Å, 250 × 4.6 mm; Phenomenex, Aschaffenburg, Germany). The analysis was performed at 30°C with 75% acetonitrile and 25% water at an isocratic flow rate of 1 ml·min⁻¹. UV detection at 195 nm was used for quantification of LNT II, GlcNAc, and pNP.

ACKNOWLEDGMENTS

Dr. Katharina Schmölzer (Graz University of Technology) is thanked for support. Dr. Melanie Weingarten (BASF SE, Ludwigshafen, Germany) kindly provided the materials for synthesis of GlcNAc-oxa. L.R. acknowledges Erasmus+ for student exchange from University of Zagreb to Graz University of Technology. J.M.B. acknowledges funding from the Government of Community of Madrid (2018-T1/BIO-10200). The thematic framework of Graz University of Technology Lead Project “PorousMaterials@Work” is acknowledged.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ORCID

Bernd Nidetzky  <http://orcid.org/0000-0002-5030-2643>

REFERENCES

- Bode, L., Contractor, N., Barile, D., Pohl, N., Prudden, A. R., Boons, G. J., ... Jennewein, S. (2016). Overcoming the limited availability of human milk oligosaccharides: Challenges and opportunities for research and application. *Nutrition Reviews*, 74, 635–644. <https://doi.org/10.1093/nutrit/nuw025>
- Bojarová, P., Bruthans, J., & Křen, V. (2019). β-N-Acetylhexosaminidases—the wizards of glycosylation. *Applied Microbiology and Biotechnology*, 103, 7869–7881. <https://doi.org/10.1007/s00253-019-10065-0>
- Bojarová, P., Kulik, N., Hovorková, M., Slámová, K., Pelantová, H., & Křen, V. (2019). The β-N-acetylhexosaminidase in the synthesis of bioactive glycans: Protein and reaction engineering. *Molecules*, 24, 599. <https://doi.org/10.3390/molecules24030599>
- Bolivar, J. M., Wiesbauer, J., & Nidetzky, B. (2011). Biotransformations in microstructured reactors: More than flowing with the stream? *Trends in Biotechnology Review*, 29, 333–342. <https://doi.org/10.1016/j.tibtech.2011.03.005>
- Chen, X. (2015). Human milk oligosaccharides (HMOS): Structure, function, and enzyme-catalyzed synthesis. *Advances in Carbohydrate Chemistry and Biochemistry*, 72, 113–190. <https://doi.org/10.1016/bs.accb.2015.08.002>
- Danby, P. M., & Withers, S. G. (2016). Advances in enzymatic glycoside synthesis. *ACS Chemical Biology*, 11, 1784–1794. <https://doi.org/10.1021/acschembio.6b00340>
- Fajjes, M., Castejon-Vilatersana, M., Val-Cid, C., & Planas, A. (2019). Enzymatic and cell factory approaches to the production of human milk oligosaccharides. *Biotechnology Advances*, 37, 667–697. <https://doi.org/10.1016/j.biotechadv.2019.03.014>
- Li, C., & Wang, L. X. (2018). Chemoenzymatic methods for the synthesis of glycoproteins. *Chemical Reviews*, 118, 8359–8413. <https://doi.org/10.1021/acs.chemrev.8b00238>
- Mateo, C., Grazu, V., & Guisan, J. M. (2013). Immobilization of enzymes on monofunctional and heterofunctional epoxy-activated supports. *Methods in Molecular Biology*, 1051, 43–57. https://doi.org/10.1007/978-1-62703-550-7_4
- Mestrom, L., Przepis, M., Kowalczykiewicz, D., Pollender, A., Kumpf, A., Marsden, S. R., ... Hagedoorn, P.-L. (2019). Leloir glycosyltransferases in applied biocatalysis: A multidisciplinary approach. *International*

- Journal of Molecular Sciences*, 20, 5263. <https://doi.org/10.3390/ijms20215263>
- Nidetzky, B., Gutmann, A., & Zhong, C. (2018). Leloir glycosyltransferases as biocatalysts for chemical production. *ACS Catalysis*, 8, 6283–6300. <https://doi.org/10.1021/acscatal.8b00710>
- Schmölzer, K., Weingarten, M., Baldenius, K., & Nidetzky, B. (2019). Glycosynthase principle transformed into biocatalytic process technology: Lacto-N-triose II production with engineered exo-hexosaminidase. *ACS Catalysis*, 9, 5503–5514. <https://doi.org/10.1021/acscatal.9b01288>
- Slámová, K., & Bojarová, P. (2017). Engineered N-acetylhexosamine-active enzymes in glycoscience. *Biochimica Biophysica Acta*, 1861, 2070–2087. <https://doi.org/10.1016/j.bbagen.2017.03.019>
- Tamborini, L., Fernandes, P., Paradisi, F., & Molinari, F. (2018). Flow bioreactors as complementary tools for biocatalytic process intensification. *Trends in Biotechnology*, 36, 73–88. <https://doi.org/10.1016/j.tibtech.2017.09.005>
- Zeuner, B., Jers, C., Mikkelsen, J. D., & Meyer, A. S. (2014). Methods for improving enzymatic trans-glycosylation for synthesis of human milk oligosaccharide biomimetics. *Journal of Agricultural and Food Chemistry*, 62, 9615–9631. <https://doi.org/10.1021/jf502619p>

- Zeuner, B., Teze, D., Muschiol, J., & Meyer, A. S. (2019). Synthesis of human milk oligosaccharides: Protein engineering strategies for improved enzymatic transglycosylation. *Molecules*, 24, 2033. <https://doi.org/10.3390/molecules24112033>

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Ruzic L, Bolivar JM, Nidetzky B. Glycosynthase reaction meets the flow: Continuous synthesis of lacto-N-triose II by engineered β -hexosaminidase immobilized on solid support. *Biotechnology and Bioengineering*. 2020;117:1597–1602. <https://doi.org/10.1002/bit.27293>