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Biocatalytic Oxidation in Continuous Flow for the Generation of Carbohydrate Dialdehydes

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ABSTRACT: Galactose Oxidase (GOase) has been used for the scalable selective C-6' oxidation of lactose, a waste material from the dairy industry. Generation of the 6'-oxo lactose was achieved with full conversion in batch mode at mg scale, but further scale-up to gram quantities proved challenging because of requirements for high enzyme concentrations and limitation in oxygen co-substrate availability. To overcome these issues, a continuous flow system was developed for the bio-oxidation of lactose yielding multi gram quantities of product. Using the variant GOase F₂, terminal selective oxidations were also observed on a range of oligoglucosides such as maltose. The carbohydrate dialdehydes that were obtained by this highly selective oxidation were chemically further functionalized establishing the biooxidation as a route to valorise cheap carbohydrates, including waste materials, for building blocks of polymers.

KEYWORDS: Galactose Oxidase, Continuous flow, Carbohydrate dialdehydes, Glucosides, Polymer.

Carbohydrates provide the greatest biomass on Earth and as such are prime candidates for renewable materials.¹⁻³ However, chemical functionalisation of carbohydrates is challenging and often requires multi-step syntheses even for simple transformations.⁴ A particularly attractive class of functional group interconversions are the selective oxidation of hydroxy groups in carbohydrates, introducing bio-orthogonal groups such as aldehydes and ketones that can then be further modified and conjugated. For example, the TEMPO-mediated oxidation of sugars with various oxidants has become one of the most popular reagents for oxidation of the primary hydroxy groups in polysaccharides (Figure 1).⁵⁻⁷ Nevertheless, TEMPO-mediated oxidation is often non-selective, leading to mixtures of products at varying oxidation states.

Therefore, enzymatic oxidations of carbohydrates have been investigated extensively.^{8,9} These biotransformations have the advantage of being in water and at ambient temperature whilst also achieving chemo- and regioselectivity without protecting groups. Galactose Oxidase (GOase) is one of the most studied biocatalysts with respect to the enzymatic oxidation of carbohydrates.⁹⁻¹¹ GOase is a copper dependent oxidase that can selectively oxidize the 6-OH position of terminal (non-reducing) D-galactose to the corresponding aldehyde whilst only requiring molecular oxygen as a co-substrate. GOase has been utilized for the synthesis of organic building blocks as well as a constituent of cascades for the synthesis of complex glycans.^{12,13} Typically GOases are strictly stereospecific with the axial orientation of the C4-hydroxyl being vital for activity; this is highlighted by the 10⁶-fold reduction in activity towards glucose. Although activity of GOase is well understood, there are very few reports of applications of this oxidase in truly scalable processes.

Flow chemistry for the synthesis of biologically relevant molecules has seen an increased use in recent years.¹⁴⁻¹⁶ Benefits that are often

cited include better control of reaction conditions, ease of scalability and increased rate of reactions.¹⁷ The use of flow in biocatalysis has also become increasingly popular, taking advantage of the ability to recycle reagents or overcome limitations from substrate inhibition.¹⁸⁻²⁰

Several reports have discussed oxygen availability for oxidase enzymes proving to be a limiting factor when scaling up a process.^{21,22} The ambient concentration of O₂ in aqueous systems is ~0.25 mM, however reports have estimated the K_{MO} for GOase variants to range between 0.5 – 5 mM.²³⁻²⁵ Furthermore, oxygen transfer limitations become more apparent on scale, underlining the limits of batch reactors for oxygen-dependent biotransformations.²⁶ As it was clear that oxygen availability could play a major role, we have investigated the use of a continuous flow reactor to improve the efficiency of oxygen-dependent biocatalysts.²¹

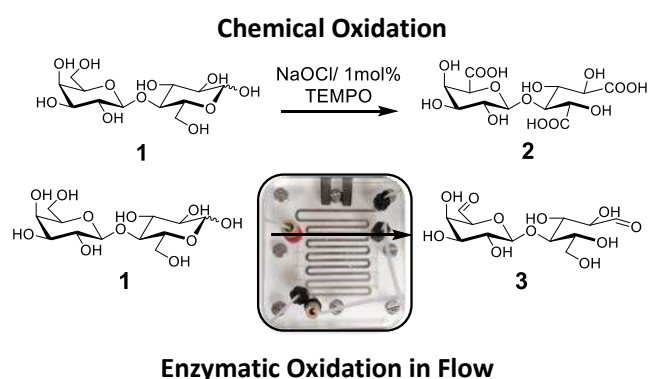


Figure 1: TEMPO-mediated chemical oxidation of C6-hydroxyl of lactose **1** leads to the triacid **2**; galactose oxidase generates **3** (shown as dialdehyde).

To demonstrate the scale-up reaction of GOase we investigated the oxidation of lactose **1** using M₁, a variant developed by Arnold and colleagues that has improved expression in *E. coli* making it a more suitable biocatalyst.^{27,28} Lactose **1** was chosen as the model substrate as it is a common waste material from the dairy industry and is responsible for the high biological oxygen demand and chemical oxygen demand of whey.²⁹ Therefore, it has been recognised as a suitable target for valorisation of waste material and the resulting dialdehyde **3** provides a potentially interesting substrate for industrial applications in bio-based materials.¹ For this reaction to be useful, quantitative oxidation is required, since separation of starting lactose **1** from product **3** is very challenging and costly.

In the first instance reactions were performed at 1 mL scale, and full conversion was achieved at up to 50 mM lactose **1** with 2 mg mL⁻¹ GOase M₁ (Supporting Information). When increasing the substrate loading to 100 mM it became apparent that the efficiency of the oxidation reduced considerably. Increase of the GOase concentration to 3 mg mL⁻¹ (keeping the substrate loading at 50mM) led to the partial over-oxidation of lactose to the corresponding carboxylic acid (Supporting Information). Though this is also a high value product for chemical synthesis, it was important to control the reaction to give homogeneous aldehyde. NMR analysis of the enzyme reaction mixture showed that under aqueous conditions the aldehyde **3** fully converts to the geminal diol with no aldehyde observed in any NMR spectra (Supporting Information).

Next, the efficiency of the oxidation on a larger scale was investigated. At 50 mM lactose **1** concentration, >99% conversion was achieved using 2.5 mg mL⁻¹ purified GOase M₁ on a 100 mL scale after 60 h incubation at 25 °C. The reaction was carried out in a 500 mL baffled flask (250 rpm). Whilst this gram scale oxidation at 50 mM was successful, the productivity (250 mg L⁻¹ h⁻¹) and efficiency (6 g_{prod} g_{enz}⁻¹) could not be improved using this standard research laboratory equipment and was considered not to be sufficient for larger scale.

Given that oxygen limitation appeared to be an issue, a previously described multi-point injection flow reactor (MPIR, Figure 2) was investigated. This set-up allows for above-ambient oxygen concentrations through *in situ* H₂O₂ degradation using catalase.²¹ Initial studies began with the GOase M₁ catalysed oxidation of lactose. The batch reactions had used GOase at a concentration of 2.5 mg mL⁻¹. As we believed the reaction to be oxygen limited, our initial conditions employed GOase at lower concentration of 1 mg mL⁻¹, the lactose at 100 mM and H₂O₂ at 100 mM final concentration, to test how well the reaction would work with increased oxygen supply (Figure 2; for full reactor details, refer to Supporting Information).

Table 1. Metrics for batch vs flow processes of enzymatic lactose oxidation

	Batch	Flow	Fold Improvement
Space time yield ^a	0.25	56	224
Efficiency ^b	6	42	7

^aSpace time yield in units of g L⁻¹ h⁻¹ ^bProductivity in units of g_{product} g_{enzyme}⁻¹

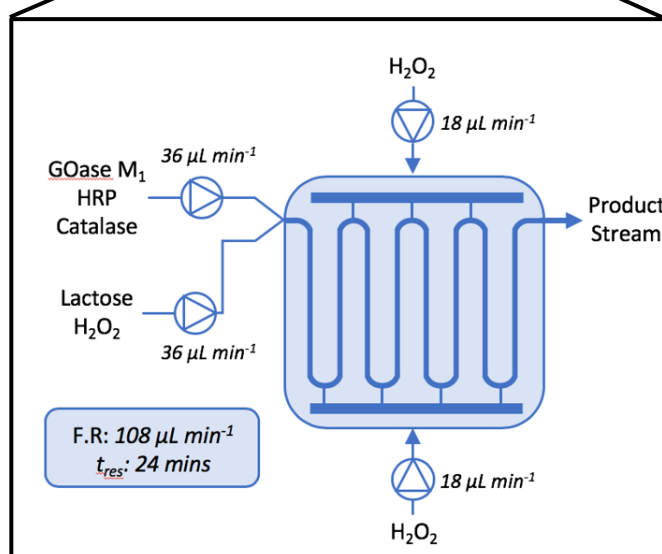


Figure 2: Schematic representation of MPIR for continuous bio-oxidation of lactose. F.R = Flow Rate; t_{res} = residence time. For concentrations refer to Figure 3.

Pleasingly, the desired product was produced at steady state with a 65% conversion. Applying a trajectory analysis recently described by Woodley and coworkers allowed us to fully optimise the process.²² Biocatalyst concentrations ranging from 0.25-2.5 mg mL⁻¹ highlighted the need for only 0.5 mg mL⁻¹ biocatalyst, with conversion always in the range of 65-70%. Increasing the H₂O₂ concentration to two molar equivalents (200 mM final conc.) proved essential, obtaining a steady state conversion of >90%, and underlining the need for the MPIR to obtain the necessary [O₂] (Figure 3, for full details of trajectory analysis see Supporting Information). To validate the effectiveness of this process, the reaction was run continuously for 20 h. Once the reaction had reached steady state the product was collected to afford a final, isolated yield of 2.10 g (85%) of **3**. This represents a space time yield of 56.7 g L⁻¹ h⁻¹ (167 mmol L⁻¹ h⁻¹), which when compared to the batch reaction (0.25 g L⁻¹ h⁻¹ (0.74 mmol L⁻¹ h⁻¹)) demonstrates a 224-fold increase in productivity gained by using *in situ* O₂ generation in the flow reactor. More importantly perhaps is the amount of enzyme used: the batch reaction required a GOase concentration of 2.5 mg mL⁻¹, whereas the flow reactor used 20% of this at 0.5 mg mL⁻¹, demonstrating a seven-fold improvement in efficiency of 42 g_{prod} g_{enz}⁻¹ with respect to the batch process (Table 1).

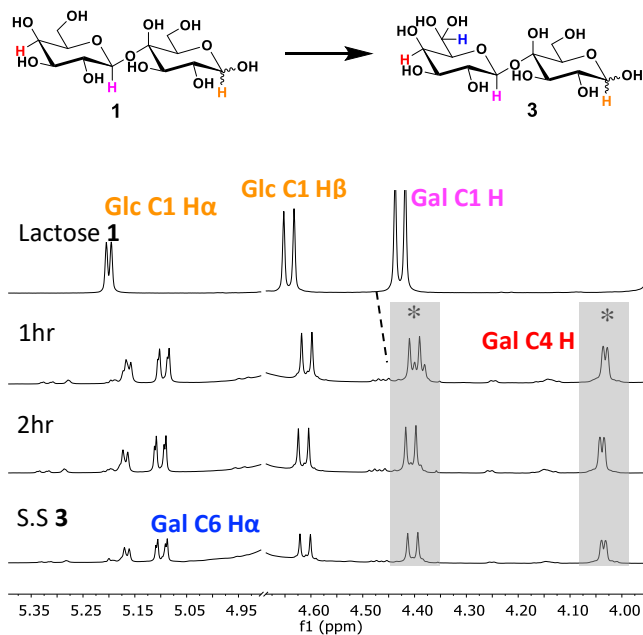


Figure 3: ^1H NMR assay used to determine 6-oxo-lactose conversion. Conversion was determined by integrating proton signals for Gal C4 and Gal C1 (S.S: flow steady state).

The application of galactose oxidase has generally been limited to terminal galactosides, but expansion to other sugars, in particular glucosides would be very desirable, given that glycosides are found in many natural polysaccharides.^{30,31} We had previously shown that mutants of galactose oxidase, in particular mutant F₂, can be used at analytical scale for the oxidation of a broader range of terminal sugars. However, the scale-up of these reaction had not been demonstrated. Several target glucosides (**4-9**, Table 2) were selected to investigate the effect of linkage and oligo length on the oxidation. In analytical scale batch reactions, GOase F₂ was incubated individually with Glc- α -OMe **4**, Glc- β -OMe **5**, Maltose **6**, Cellobiose **7**, Maltotriose **8** and Cellotriose **9** for 16 h (Table 2). Analysis of the ^1H NMR spectra showed that GOase F₂ displayed activity against all glucosides tested with varied conversion. For comparison, GOase M₁ showed no activity towards any of the tested glucosides. As with lactose, the respective geminal diols (**10-15**) were observed by HRMS and ^1H NMR analysis (Supporting Information).

Following on from the glucoside screening, maltose (**6**) was chosen as the substrate to investigate further as it is a grain based renewable feedstock. In batch, only 15% conversion was observed with 100 mM maltose at 1 mg mL⁻¹ enzyme concentration after 24 h. Applying the optimised conditions for lactose in flow for maltose, at 100 mM substrate concentration steady state conversion was observed at 55% within 2 h (see Supporting Information). The lower activity of the F₂ variant limited the reaction and further enzyme engineering will be required to further optimise the reaction. However these results demonstrate the benefits the M₁ can offer across GOase variants for carbohydrate functionalisation.

The synthetic potential for carbohydrate-based polymers is well documented.^{1,32,33} To demonstrate the potential bio-material applications of the resulting lactodialdehyde **3** (in equilibrium with the ring-closed form of **3**, but shown here to be in the open form for clarity) as a precursor for a potential biomaterial, the use of bis-hydroxylamines as linkers was investigated for the production of lactose-based polymers (Figure 4).^{34,35}

Substrate	Product	% Conversion
4	10	60
5	11	40
6	12	62
7	13	27
8	14	>99
9	15	81

Conditions: glucoside (20 mM), GOase F₂ (1 mg mL⁻¹), HRP (0.1 mg mL⁻¹), catalase (0.1 mg mL⁻¹), 25 °C, 250 rpm, 16 h; conversions determined by ^1H -NMR.

The reaction product was therefore analysed by MALDI-ToF (Figure 4B). Mass spec analysis clearly showed the presence of the monomer in addition to the dimer, trimer and tetramer. After leaving the reaction to proceed for 24 h, a film-like precipitate was observed (Figure 4C) suggesting successful formation of higher polymeric material. Comparison of the infra red spectra of the dialdehyde and the film showed a shift in the characteristic signals, with a signal at 1637 cm⁻¹ in the product likely attributable to the formation of the oxime functional groups (see supporting information). ^1H NMR analysis was challenging as the resulting oxime has E/Z isomers in the ring closed form at the anomeric position, a problem previously reported by Feizi and colleagues.³⁵ In addition, efforts to obtain ^1H NMR spectra were hindered by the insolubility of the product in multiple deuterated solvents.

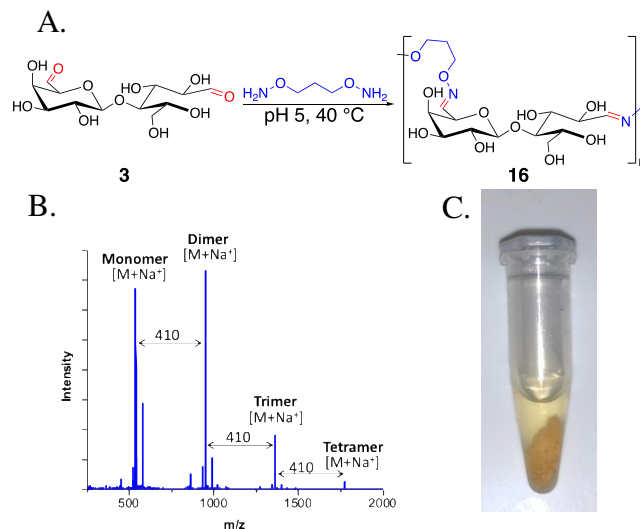


Table 2: Oxidations of Glucosides with GOase F₂

Figure 4: A. Generation of lactose based-oxime polymers using dihydroxylamine linkers B. MALDI-ToF analysis of soluble aliquot from condensation reaction C. Polymerisation reaction that was left overnight lead to generation of a film-like precipitate.

In summary, we have described a biocatalytic process which has been applied in the regioselective oxidation of several saccharides including lactose and maltose using engineered GOase variants. Using standard lab 'batch' equipment, this process suffered from lack of availability of molecular oxygen to the reaction. Utilizing a continuous flow reactor we were able to overcome this issue to achieve efficient bio-oxidations to produce 6-oxo-lactose, in multi-gram quantities. The same system was then also used with the variant F₂ and maltose. It is envisaged that with further engineering of GOase, alongside improvements in reactor design, this would provide a reliable and scalable method for selective terminal carbohydrate functionalisation. Finally, proof-of-concept studies have demonstrated that polymerisation of the oxidised lactose product is possible, offer opportunities for new sugar-based polymer materials in future.

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Supporting Information

Materials, experimental procedures, characterization of new compounds. This material is available free of charge via the internet at <http://pubs.acs.org>

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The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. / ‡These authors contributed equally.

Notes

The authors declare no competing financial interests.

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REFERENCES

- (1) Galbis, J. A.; García-Martín, M. de G.; De Paz, M. V.; Galbis, E. Synthetic Polymers from Sugar-Based Monomers. *Chem. Rev.* **2016**, *116*, 1600–1636.
- (2) Kabasci, S. *Bio-Based Plastics: Materials and Applications*; Kabasci, S., Ed.; John Wiley & Sons Ltd: Chichester, UK, 2014.
- (3) Bozell, J. J.; Petersen, G. R. Technology Development for the Production of Biobased Products from Biorefinery Carbohydrates - The US Department of Energy's "Top 10" Revisited. *Green Chem.* **2010**, *12*, 539–554.
- (4) Jäger, M.; Minnaard, A. J. Regioselective Modification of Unprotected Glycosides. *Chem. Commun.* **2016**, *52*, 656–664.
- (5) Davis, N. J.; Flitsch, S. L. Selective Oxidation of Monosaccharide Derivatives to Uronic Acids. *Tetrahedron Lett.* **1993**, *34*, 1181–1184.
- (6) de Nooy, A. E. J.; Besemer, A. C.; van Bekkum, H. Selective Oxidation of Primary Alcohols Mediated by Nitroxyl Radical in Aqueous Solution. Kinetics and Mechanism. *Tetrahedron* **1995**, *51*, 8023–8032.
- (7) Breton, T.; Bashiardes, G.; Leger, J. M.; Kokoh, K. B. Selective Oxidation of Unprotected Carbohydrates to Aldehyde Analogues by Using TEMPO Salts. *European J. Org. Chem.* **2007**, *10*, 1567–1570.
- (8) Raba, J.; Mottola, H. A. Glucose Oxidase as an Analytical Reagent. *Crit. Rev. Anal. Chem.* **1995**, *25*, 1–42.
- (9) Parikka, K.; Master, E.; Tenkanen, M. Oxidation with Galactose Oxidase: Multifunctional Enzymatic Catalysis. *J. Mol. Catal. B Enzym.* **2015**, *120*, 47–59.
- (10) Rannes, J. B.; Ioannou, A.; Willies, S. C.; Grogan, G.; Behrens, C.; Flitsch, S. L.; Turner, N. J. Glycoprotein Labeling Using Engineered Variants of Galactose Oxidase Obtained by Directed Evolution. *J. Am. Chem. Soc.* **2011**, *133*, 8436–8439.
- (11) Whittaker, J. W. Galactose Oxidase. In *Advances in Protein Chemistry*; Elsevier; Amsterdam; 2002; Vol. 60, pp 1–49.
- (12) Kupper, C. E.; Rosencrantz, R. R.; Henßen, B.; Pelantová, H.; Thönes, S.; Drozdová, A.; Kren, V.; Elling, L. Chemo-Enzymatic Modification of Poly-N-Acetylglucosamine (LacNAc) Oligomers and N,N-Diacetyllactosamine (LacDiNAc) Based on Galactose Oxidase Treatment. *Beilstein J. Org. Chem.* **2012**, *8*, 712–725.
- (13) Escalettes, F.; Turner, N. J. Directed Evolution of Galactose Oxidase: Generation of Enantioselective Secondary Alcohol Oxidases. *ChemBioChem* **2008**, *9*, 857–860.
- (14) Plutschack, M. B.; Pieber, B.; Gilmore, K.; Seeberger, P. H. The Hitchhiker's Guide to Flow Chemistry. *Chem. Rev.* **2017**, *117*, 11796–11893.
- (15) Blacker, A. J.; Breen, J. R.; Bourne, R. A.; Hone, C. A. The Growing Impact of Continuous Flow Methods on the Twelve Principles of Green Chemistry. *Green Sustain. Med. Chem. Methods Tools Strateg. 21st Century Pharm. Ind.*; Royal Society of Chemistry; Cambridge; 2016, No. 46, pp 140–155.
- (16) Pastre, J. C.; Browne, D. L.; Ley, S. V. Flow Chemistry Syntheses of Natural Products. *Chem. Soc. Rev.* **2013**, *42*, 8849–8869.
- (17) Dallinger, D.; Kappe, C. O. Why Flow Means Green – Evaluating the Merits of Continuous Processing in the Context of Sustainability. *Curr. Opin. Green Sustain.*

- Chem.* **2017**, *7*, 6–12.
- (18) Britton, J.; Jamison, T. F. The Assembly and Use of Continuous Flow Systems for Chemical Synthesis. *Nat. Protoc.* **2017**, *12*, 2423–2446.
- (19) Thompson, M. P.; Peñafiel, I.; Cosgrove, S. C.; Turner, N. J. Biocatalysis Using Immobilized Enzymes in Continuous Flow for the Synthesis of Fine Chemicals. *Org. Process Res. Dev.* **2019**, *23*, 9–18.
- (20) Yuryev, R.; Stropfen, S.; Liese, A. Coupled Chemo(Enzymatic) Reactions in Continuous Flow. *Beilstein J. Org. Chem.* **2011**, *7*, 1449–1467.
- (21) Chapman, M. R.; Cosgrove, S. C.; Turner, N. J.; Kapur, N.; Blacker, A. J. Highly Productive Oxidative Biocatalysis in Continuous Flow by Enhancing the Aqueous Equilibrium Solubility of Oxygen. *Angew. Chem. Int. Ed.* **2018**, *57*, 10535–10539.
- (22) Nordblad, M.; Gomes, M. D.; Meissner, M. P.; Ramesh, H.; Woodley, J. M. Scoping Biocatalyst Performance Using Reaction Trajectory Analysis. *Org. Process Res. Dev.* **2018**, *22*, 1101–1114.
- (23) Kwiatkowski, L. D.; Adelman, M.; Pennelly, R.; Kosman, D. J. Kinetic Mechanism of the Cu(II) Enzyme Galactose Oxidase. *J. Inorg. Biochem.* **1981**, *14*, 209–222.
- (24) Humphreys, K. J.; Mirica, L. M.; Wang, Y.; Klinman, J. P. Galactose Oxidase as a Model for Reactivity at a Copper Superoxide Center. *J. Am. Chem. Soc.* **2009**, *131*, 4657–4663.
- (25) Toftgaard Pedersen, A.; Birmingham, W. R.; Rehn, G.; Charnock, S. J.; Turner, N. J.; Woodley, J. M. Process Requirements of Galactose Oxidase Catalyzed Oxidation of Alcohols. *Org. Process Res. Dev.* **2015**, *19*, 1580–1589.
- (26) Meissner, M. P.; Nordblad, M.; Woodley, J. M. Online Measurement of Oxygen-Dependent Enzyme Reaction Kinetics. *ChemBioChem* **2018**, *19*, 106–113.
- (27) Sun, L.; Bulter, T.; Alcalde, M.; Petrounia, I. P.; Arnold, F. H. Modification of Galactose Oxidase to Introduce Glucose 6-Oxidase Activity. *ChemBioChem* **2002**, *3*, 781–783.
- (28) Sun, L.; Petrounia, I. P.; Yagasaki, M.; Bandara, G.; Arnold, F. H. Expression and Stabilization of Galactose Oxidase in Escherichia Coli by Directed Evolution. *Protein Eng.* **2001**, *14*, 699–704.
- (29) Nath, A.; Verasztó, B.; Basak, S.; Koris, A.; Kovács, Z.; Vatai, G. Synthesis of Lactose-Derived Nutraceuticals from Dairy Waste Whey—a Review. *Food Bioprocess Technol.* **2016**, *9*, 16–48.
- (30) Kunamneni, A.; Plou, F. J.; Alcalde, M.; Ballesteros, A. Trichoderma Enzymes for Food Industries. In *Biotechnology and Biology of Trichoderma*; Elsevier: Amsterdam; 2014; pp 339–344.
- (31) van Wijk, A.; Siebum, A.; Schoevaart, R.; Kieboom, T. Enzymatically Oxidized Lactose and Derivatives Thereof as Potential Protein Cross-Linkers. *Carbohydr. Res.* **2006**, *341*, 2921–2926.
- (32) Rose, M.; Palkovits, R. Cellulose-Based Sustainable Polymers: State of the Art and Future Trends. *Macromol. Rapid Commun.* **2011**, *32*, 1299–1311.
- (33) Delidovich, I.; Hausoul, P. J. C.; Deng, L.; Pfützenreuter, R.; Rose, M.; Palkovits, R. Alternative Monomers Based on Lignocellulose and Their Use for Polymer Production. *Chem. Rev.* **2016**, *116*, 1540–1599.
- (34) Collins, J.; Xiao, Z.; Müllner, M.; Connal, L. A. The Emergence of Oxime Click Chemistry and Its Utility in Polymer Science. *Polym. Chem.* **2016**, *7*, 3812–3826.
- (35) Liu, Y.; Feizi, T.; Campanero-Rhodes, M. A.; Childs, R. A.; Zhang, Y.; Mulloy, B.; Evans, P. G.; Osborn, H. M. I.; Otto, D.; Crocker, P. R.; Chai, W. Neoglycolipid Probes Prepared via Oxime Ligation for Microarray Analysis of Oligosaccharide-Protein Interactions. *Chem. Biol.* **2007**, *14*, 847–859.

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