Research article

Characterization and immobilization of engineered sialidases from *Trypanosoma rangeli* for transsialylation

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Abstract: A sialidase (EC 3.2.1.18; GH 33) from non-pathogenic *Trypanosoma rangeli* has been engineered with the aim of improving its transsialylation activity. Recently, two engineered variants containing 15 and 16 amino acid substitutions, respectively, were found to exhibit significantly improved transsialylation activity: both had a 14 times higher ratio between transsialylation and hydrolysis products compared to the first reported mutant TrSA$_{5mut}$. In the current work, these two variants, Tr15 and Tr16, were characterized in terms of pH optimum, thermal stability, effect of acceptor-to-donor ratio, and acceptor specificity for transsialylation using casein glycomacropeptide (CGMP) as sialyl donor and lactose or other human milk oligosaccharide core structures as acceptors. Both sialidase variants exhibited pH optima around pH 4.8. Thermal stability of each enzyme was comparable to that of previously developed *T. rangeli* sialidase variants and higher than that of the native transsialidase from *T. cruzi* (TcTS). As for other engineered *T. rangeli* sialidase variants and TcTS, the acceptor specificity was broad: lactose, galactooligosaccharides (GOS), xylooligosaccharides (XOS), and human milk oligosaccharide structures lacto-*N*-tetraose (LNT), lacto-*N*-fucopentaose (LNFP V), and lacto-*N*-neofucopentaose V (LNnFP V) were all sialylated by Tr15 and Tr16. An increase in acceptor-to-donor ratio from 2 to 10 had a positive effect on transsialylation. Both enzymes showed high preference for formation α(2,3)-linkages at the non-reducing end of lactose in the transsialylation. Tr15 was the most efficient enzyme in terms of transsialylation reaction rates and yield of 3'-sialyllactose. Finally, Tr15 was immobilized covalently on glyoxyl-functionalized silica, leading to a 1.5-fold increase in biocatalytic productivity (mg 3'-sialyllactose per mg

enzyme) compared to free enzyme after 6 cycles of reuse. The use of glyoxyl-functionalized silica proved to be markedly better for immobilization than silica functionalized with (3-aminopropyl)triethoxysilane (APTES) and glutaraldehyde, which resulted in a biocatalytic productivity which was less than half of that obtained with free enzyme.

Abbreviations:

3'SL: 3'-sialyllactose

6'SL: 6'-sialyllactose

APTES: (3-aminopropyl)triethoxysilane

GA: glutaraldehyde

GPTMS: (3-glycidyloxypropyl)trimethoxysilane

LNFP V: lacto-*N*-fucopentaose V

LNnT: lacto-*N*-neotetraose

SA: sialic acid

TcTS: transsialidase from *Trypanosoma cruzi*

TrSA: sialidase from *Trypanosoma rangeli*

XOS: xylooligosaccharides

3SL: 3-sialyllactose

A:D: acceptor-to-donor ratio

CGMP: casein glycomacropeptide

GOS: galactooligosaccharides

HMO: human milk oligosaccharides

LNnFP V: lacto-*N*-neofucopentaose V

LNT: lacto-*N*-tetraose

SL: sialyllactose

THF: tetrahydrofuran

TrX: TrSA with X amino acid substitutions

1. Introduction

Human milk oligosaccharides (HMOs) designate a unique family of bioactive lactose-based molecules present in human breast milk (5–15 g/L), which are known to be of major importance for infant health and development [1]. However, as they are virtually absent from bovine milk, which is used for production of infant formula, production of HMOs receives increasing attention for commercial use as well as for functional studies. The concentration of sialylated HMOs is around 1.8 g/L, corresponding to 19% (w/w) of the HMOs for secretors and as much as 33% (w/w) for non-secretors [2]. The sialylated HMOs are believed to stimulate the immune system, increase the resistance of the breast-fed infant towards pathogens, assist in gut maturation and microbiota development, as well as play a role in the brain development of the infant [1,3].

One of the few native transsialidases, the transsialidase from the human pathogen *Trypanosoma cruzi* (TcTS) has high transsialidase activity and extremely low hydrolytic activity, and has thus been used for efficient enzymatic preparation of sialylated glycans [4-7]. However, for industrial production of food-grade HMOs, the fact that TcTS is an important virulence factor in *T. cruzi* is an indisputable obstacle [8]. Consequently, major efforts have been put into engineering the sialidase from non-pathogenic *T. rangeli* (TrSA) into an efficient sialidase by substituting amino residues in TrSA by the corresponding ones found in TcTS [9,10]. Indeed, TrSA has 70% sequence identity to TcTS and the same overall structure, but no transsialidase activity [11,12]. Despite their similarity, it has become evident that it is not a simple task to identify the amino acids required for efficient transsialidase activity [9,10,13,14]. Introducing five mutations affecting the binding site and the sialic acid binding

pocket in TrSA conferred a weak transsialidase activity in the resulting enzyme, TrSA$_{5mut}$ [9]. Either of two additional mutations increased the transsialidase activity to 11% of that of TcTS, but also increased k_{cat}/K_m of the hydrolysis of the desired product, 3'-sialyllactose (3'SL), 1.8-fold [9]. The resulting enzyme, Tr6, has subsequently been characterized in terms of optimal reaction conditions and thermal stability, used for gram scale production of 3'SL, immobilized, and recycled [15,16]. Additional substitution of a seven amino acid loop located near the acceptor binding site reduced the hydrolytic activity 4-fold without affecting the transsialidase activity. The mutation, which was based on the corresponding loop found in TcTS, introduced a net charge of +3 which was hypothesized to cause partial reversal of the water network in the active site, thus impairing the nucleophilicity of the water [10]. The resulting engineered sialidase, Tr13, was characterized in terms of optimal reaction conditions and thermal stability, used for gram scale production of sialylated galactooligosaccharides (GOS), and recycled in an enzymatic membrane reactor [10,17]. Finally, a redesign of TrSA$_{5mut}$ by introducing five additional mutations identified by free energy computations was suggested, but not validated experimentally [14].

Recently, the many studies on how to confer transsialidase activity on TrSA were integrated into a systematic study including seven expressed mutants, which were investigated for transsialidase and sialidase activity using casein glycomacropeptide (CGMP) as donor and lactose as acceptor [18]. This led to identification of two novel mutants with improved transsialidase activity and reduced hydrolase activity, having 15 and 16 substitutions in the polypeptide backbone, respectively. The mutants were named TrSA$_{15mut}$ (Tr15) and TrSA$_{16mut}$ (Tr16) [18]. Tr16 is an integration of Tr13 [10] and the three "energetic mutations" T39A, F59N, and D285G, that based on energy decomposition analysis were predicted by theoretical modeling to lower the stability of the covalent intermediate (CI) thus promoting transsialidase activity [14] (Table 1). Tr15 is identical to Tr16 except for the lack of the "structural mutation" I37L, which was hypothesized to increase the participation of the nucleophile at the transition state by improving the mobility of the nucleophile [14] (Table 1). The I37L mutation improved the formation of transsialylation products, but it also reduced the overall activity significantly [18].

Table 1. Mutations introduced in the sialidase from *Trypanosoma rangeli* (TrSA) to create the engineered sialidases used in the current study. In all cases, the residues were mutated to the corresponding residue in the transsialidase from *Trypanosoma cruzi* (TcTS), yielding Tr6 [9], Tr13 [10], Tr15 and Tr16 [18], where the number indicates the number of mutations. Amino acid residue numbering is based on TrSA.

Mutation groups	Mutations	Tr6	Tr13	Tr15	Tr16
TrSA$_{5mut}$ [9]	M96V, A98P, S120Y, G249Y, Q284P	X	X	X	X
Structural [9,14]	I37L	X	X		X
Energetic [14]	T39A, F59N, D285G			X	X
Loop [10]	I197V, A198T, D199N, M200K, G201K, G202K, R203Q		X	X	X

As part of the current work, the amino acid substitutions introduced in TrSA to create Tr16 were visualized in a homology model created using the crystal structure of TrSA (PDB: 1MS9) as a template (Figure 1). The trisaccharide 3'SL, which can be a product of transsialylation activity as well as a substrate for the sialidase activity, was positioned in the active site by aligning the homology model to a crystal structure of TcTS having 3'SL as a ligand (PDB: 1S0I).
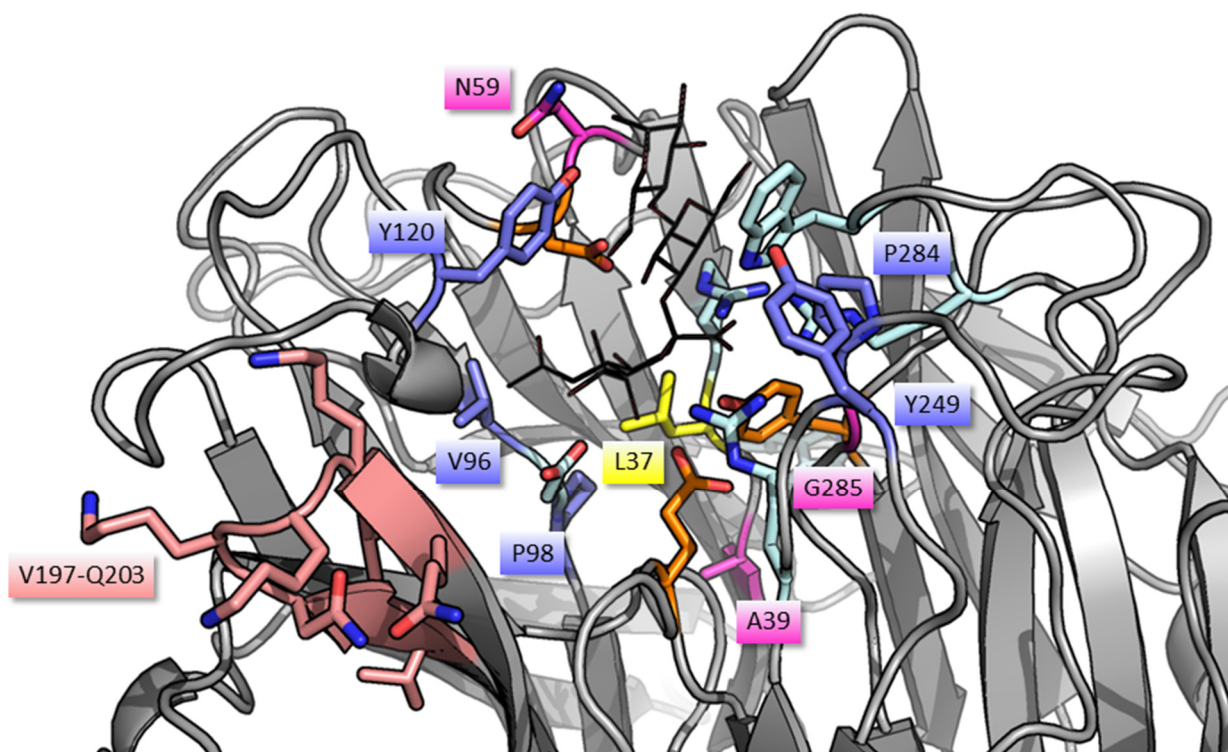
Figure 1. Homology model of Tr16 (grey; modelled with HHPred and MODELLER using 1MS9 as template) illustrating the position of the introduced amino acid substitutions (Table 1) around the active site, where 3'SL is bound (black lines; from 1S0I). Amino acid substitutions: TrSA$_{5mut}$ (purple), loop (salmon), structural (yellow), energetic (pink). Catalytic residues (D60, E231, Y343; orange) and other strongly interacting residues not substituted (R36, D97, R246, W313, R315; light blue; [49]) are also shown.

When using a glycosidase to catalyze transglycosylation, the reaction is a kinetically controlled synthesis [19]. The synthetic reaction (transglycosylation) takes place in competition with hydrolysis of both substrate and product, named primary and secondary hydrolysis, respectively (Figure 2). The ratio between the transglycosylation rate (r_T) and the hydrolysis rate (r_H) describes how efficiently the glycosyl-enzyme intermediate gives the desired transglycosylation products (Figure 2). Thus, in order to maximize transglycosylation yields the ratio r_T/r_H must be maximized, and [19-21]:

$$\frac{r_T}{r_H} = S \cdot \frac{a_{glycosyl\ acceptor}}{a_w} = S_c \cdot \frac{[glycosyl\ acceptor]}{[water]} \qquad \textbf{Equation 1}$$

In essence, the transglycosylation yield is influenced by the activities (or concentrations) of the competing nucleophiles as well as by the selectivity factor S (or S_c), which is dependent on the intrinsic properties of the enzyme and also on the choice of donor, acceptor and reaction conditions. It follows from the set of reactions (Figure 2), that the maximum yield will be transient unless the secondary hydrolysis is negligible [19-21].
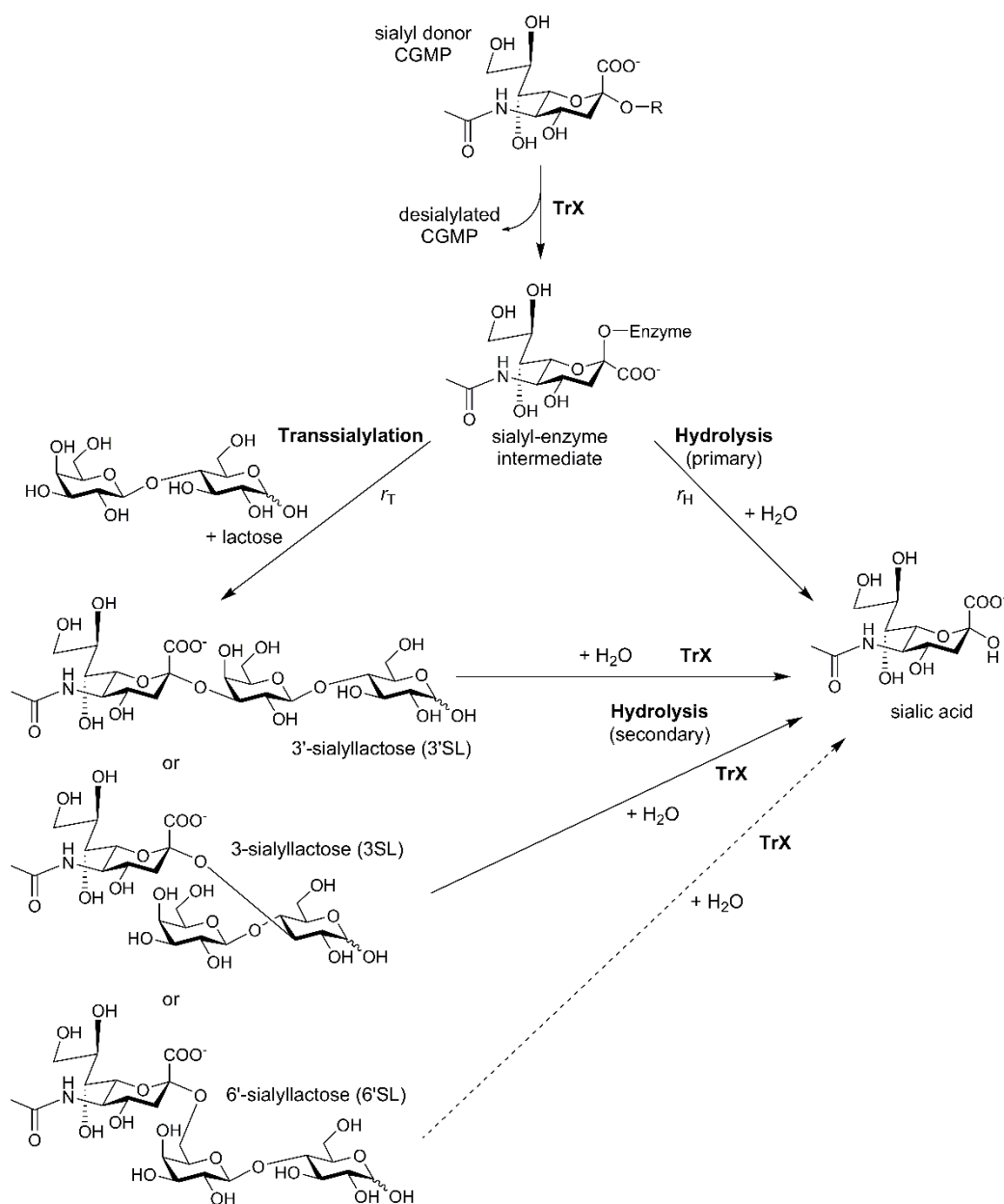
Figure 2. Reactions catalyzed by the *T. rangeli* sialidase variants (TrX) when using casein glycomacropeptide (CGMP) as sialyl donor and lactose as acceptor. The sialidases (GH 33) follow a retaining double displacement mechanism [57], where the nucleophilic attack on the sialyl-enzyme intermediate will either lead to transglycosylation or (primary) hydrolysis. The ratio between the transglycosylation rate (r_T) and the hydrolysis rate (r_H) is indicative of the transglycosylation performance, and is determined by the properties of the enzyme, donor and acceptor substrates, as well as by the reaction conditions [16,19,20]. The regioselectivity of the *T. rangeli* sialidase variants favors formation of 3'-sialyllactose (3'SL), but some 3-sialyllactose (3SL) and, in some cases, trace amounts of 6'-sialyllactose (6'SL) are also formed (Figure 4). 3'SL and 3SL are also substrates for TrX and may thus undergo (secondary) hydrolysis, resulting in a transient maximum yield—a characteristic of kinetically controlled synthesis [19,20]. 6'SL was not a substrate for Tr6 and Tr13 [10]. 3'SL and 6'SL are known HMOs, while 3SL is not. Lactose is used as an example of an acceptor here; other possible acceptors are given in Table 4.

The aim of this paper is to characterize the two superior engineered *T. rangeli* sialidases, Tr15 and Tr16, and notably to compare different enzyme immobilization strategies in order to accomplish efficient, enzymatic transsialylation. It was hypothesized that Tr15 and Tr16 would be more efficient than their predecessors $TrSA_{6mut}$ (Tr6) and $TrSA_{13mut}$ (Tr13) in transsialylation at low acceptor-to-donor ratios—a prerequisite for working with transsialylation of expensive acceptor substrates such as lacto-*N*-tetraoses, which is often neglected because the sialidase-catalyzed transsialylation is enhanced by high acceptor-to-donor ratios [22]. In this context, acceptor specificity of Tr15 and Tr16 was also assessed.

The biocatalytic productivity of an enzyme, i.e. amount of product per amount of enzyme, can be improved by enzyme immobilization as it facilitates recycling of the enzyme. Furthermore, immobilization may increase enzyme stability, activity, specificity, and/or selectivity and may also reduce inhibition effects by the medium or products [23,24]. The use of porous supports can generate favourable microenvironments leading to an improvement of enzyme performance. Porous supports may promote desirable diffusional limitations such as substrate/product concentration gradients or pH gradients, have protective effects on the confined enzyme, or block certain areas which in turn prevents enzyme inhibition [24]. Covalent immobilization is the most widely applied method in industrial applications due to several advantages including improved operational stability, robustness and reusability. Glutaraldehyde is a popular and versatile reagent in protein immobilization because although it normally reacts with primary amino groups in the protein, it can also react with thiol, phenol and imidazole groups. The mechanisms behind glutaraldehyde-mediated immobilization are not fully understood, but it is clear that the mechanisms differ depending on the pH [25]. At neutral and acidic conditions as employed in the current work, the cyclic hemiacetal forms of both monomeric and polymeric glutaraldehyde would react by nucleophilic substitution amino groups on the protein surface of the enzyme with the hydroxyl group of glutaraldehyde [25]. At neutral or acidic pH, the most reactive amino group is the terminal one with a pK_a value of 7.7 compared to the pK_a value of 10.5 of the ε-amino groups of Lys residues and it is thus likely that the first covalent bond is formed with the N-terminal [25,26]. However, when the enzyme is thus brought into proximity with glutaraldehyde moieties on the support, it is likely that further covalent enzyme-support bonds will be formed if other nucleophiles on the enzyme are present on the side facing the support. Alternatively, the enzyme can be adsorbed to the support through hydrophobic interaction once the unipoint covalent linkage to glutaraldehyde is formed [25]. Enzyme immobilization on glyoxyl-functionalized supports establishes multipoint covalent bonds to several reactive primary amines, mainly ε-amino groups of surface Lys residues. Immobilization must take place at alkaline conditions (pH 10) and requires a highly activated support. This kind of immobilization occurs through more than one simultaneous enzyme-support interaction. In fact, the density of reactive groups is a driving force, and as a consequence immobilization takes place in the area(s) where the enzyme has the highest density of Lys residues [27]. A final step is the reduction with sodium borohydride of Schiff's bases between amino and glyoxyl groups into stable secondary amino bonds and of all remaining glyoxyl (aldehyde) groups into inert, hydrophilic hydroxyl groups [27-29]. Glyoxyl-functionalized agarose or silica supports permit a fair enzyme stabilization and reuse without leakage of the enzyme into the medium [23,27]. Agarose support has been widely used with successful results [30,31]. Alternatively, silica supports with porous structures offer several advantages as high surface area, non-toxicity, microbe resistance, ease of handling, and robustness towards high flow rates in continuous reactors and have been used successfully for glyoxyl immobilization of β-galactosidases [32-34].

Recently, the commercial crude extract Pectinex Ultra SP-L, which includes β-galactosidase from *Aspergillus aculeatus*, was immobilized on a glyoxyl-functionalized porous silica support with a wide pore size [35]. The immobilized biocatalyst demonstrated an improved yield of galactooligosaccharides (GOS) compared to the use of free enzyme. Furthermore, the ratio between transgalactosylation and hydrolysis was improved by immobilization. This was probably due to a combination of proper hydrophobic/hydrophilic surface balance and a confinement effect of substrates and products inside the pores of the support [35]. As β-galactosidases and sialidases share a similar reaction mechanism [22] (Figure 2), it was hypothesized that transsialylation catalyzed by the engineered *T. rangeli* sialidases would benefit from immobilization on porous silica support grafted with glyoxyl [35]. For comparison, the sialidases were immobilized in the same porous silica support grafted with (3-aminopropyl)triethoxysilane (APTES) and glutaraldehyde (GA). The APTES-GA method is the most popular route to covalent immobilization of biomolecules including enzymes on silica supports [32,36-38], and therefore serves as a good comparison to the glyoxyl method. Additionally, two methods of recycling, centrifugation and fed-batch, were evaluated for all the biocatalysts synthesized. It was hypothesized that enzyme immobilization would improve the biocatalytic productivity in the transsialylation reaction and that its covalent nature would result in less leakage of the enzyme from the support than observed previously for Tr6 [15]. In that study, the most successful method, immobilization on Cu^{2+}-iminodiacetic acid-functionalized magnetic nanoparticles *via* the His-tag, resulted in a reduction in transsialylation activity to as little as 14% in just four 1-hour reaction cycles, mainly due to enzyme leakage [15].

2. Materials and Methods

2.1. Chemicals

3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL) were purchased from Carbosynth Ltd (Compton, UK). Casein glycomacropeptide (CGMP), in the form of the commercial product Lacprodan CGMP-20, and galactooligosaccharides (GOS) 570-P (product of King-Prebiotics, Yunfu, China) were kindly provided by Arla Foods Ingredients Group (Viby, Denmark). GOS Cup Oligo P (Nissin Sugar Co., Tokyo, Japan) were purchased from Giulio Gross (Trezzano sul Naviglio, Italy). Xylooligosaccharides (XOS) from Longlive Biotechnology (Shandong, China) were a gift from Dr. Arthur Ouwehand (DuPont Nutrition & Health, Kantvik, Finland). Silica support Sipernat 50S was kindly donated by Evonik Resource Efficiency GmbH (Essen, Germany). Glutaraldehyde was kindly provided by Novozymes A/S (Bagsværd, Denmark). Glycerol was purchased from VWR (Leuven, Belgium) and tetrahydrofuran (THF) from Fisher Scientific (Loughborough, UK). Lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT), lacto-*N*-fucopentaose V (LNFP V), and lacto-*N*-neofucopentaose V (LNnFP V) were purchased from Elicityl Oligotech (Crolles, France). All other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Enzyme production and purification

Codon-optimized gene constructs of Tr6 and Tr13 for expression in *Pichia pastoris* were synthesized by DNA 2.0 (Menlo Park, CA, USA), and further mutations were introduced using the QuikChange II Site-Directed Mutagenesis Kit (Agilent, CA, USA) and mutagenic primers [18]. For

high cell density fermentation of His$_6$-tagged Tr6, Tr13, Tr15, and Tr16, *P. pastoris* X-33 strains each harboring pPICZαC with the particular mutated enzyme gene was fermented in a 5 L Sartorius Biostat Aplus fermentor as described previously [16]. Purification of the His$_6$-tagged enzymes was performed by Ni^{2+} affinity column chromatography as described previously [15]. Protein concentrations were determined by the BCA protein assay kit (Thermo Scientific, Rockford, USA) with bovine serum albumin as external standard.

2.3. Thermal stability and pH optimum

Thermal stability of Tr15 and Tr16 was determined by incubation of the enzymes at 30, 40, 45, 50, and 55 °C for relevant times up to 2 hours, followed by determination of residual transsialidase activity. The pH optima of Tr15 and Tr16 were determined at 30 °C in 160 mM phosphate-citrate buffer solutions over a resulting pH range from 3.9 to 7.7, where the exact pH was measured in each sample; the uneven numbers were caused by the large buffering effect of CGMP, emphasizing the need for measuring the exact pH in the sample. In both cases, transsialidase activity was assessed using 34 g/L CGMP (corresponding to 8 mM bound sialic acid) as donor substrate, 80 mM β-lactose as acceptor, and an enzyme concentration of 6 mg/L. For the thermal stability tests, the reaction time was 20 min, whereas it was 10 min for Tr15 and 30 min for Tr16 in the pH optimum studies. The reaction was terminated by heat enzyme inactivation at 90 °C for 10 min. The samples were filtered on a 5 kDa polyethersulfone membrane (Vivaspin 500; Sartorius AG, Göttingen, Germany) to remove CGMP before the concentrations of sialyllactose (SL) were determined by HPAEC-PAD (see below).

2.4. Time study at varying acceptor-to-donor ratio

The effect of acceptor-to-donor (A:D) ratio was evaluated for Tr13, Tr15, and Tr16 in a time study, where the reaction was monitored for 3 hours. The reactions were run at 30 °C in 160 mM phosphate-citrate buffer solution at resulting optimal pH (pH 5.5 for Tr13 [17], and pH 4.8 for Tr15 and Tr16), using 34 g/L CGMP (corresponding to 8 mM bound sialic acid [17]) as donor substrate, 16 or 80 mM β-lactose as acceptor (A:D = 2 or A:D = 10, respectively), and an enzyme concentration of 6 mg/L. Reactions were stopped at relevant time points between 0 min and 3 hours and prepared for HPAEC-PAD analysis as above. Since the transglycosylation yield is influenced by product hydrolysis, only initial rates of substrate hydrolysis and transglycosylation product formation were considered when calculating reaction rates r_T and r_H.

2.5. Transsialylation of LNT, LNnT, LNFP V, LNnFP V, GOS, and XOS

For transsialylation of alternative acceptors LNT, LNnT, LNFP V, LNnFP V, XOS, and GOS with Tr15 and Tr16, the reaction took place for up to 4 hours at 30 °C in 160 mM acetate buffer at pH 4.8, using 34 g/L CGMP (corresponding to 8 mM bound sialic acid) as donor substrate, 80 mM LNT, LNnT, LNFP V, or LNFP V or 27.5 g/L GOS or XOS as acceptor, and an enzyme concentration of 6 mg/L. Negative controls were performed with heat-inactivated enzyme. The reaction was terminated by heat enzyme inactivation at 90 °C for 10 min. For transsialylations of LNT, LNnT, LNFP V, and LNnFP V, the samples were filtered on a 5 kDa polyethersulfone membrane (Vivaspin 500; Sartorius AG, Göttingen, Germany) to remove CGMP. For all GOS or XOS transsialylations, the heat-

inactivated reaction mixture was subjected to anion exchange chromatography on a HiTrap Q FF column (GE Healthcare, Uppsala, Sweden) as described previously [17] in order to purify the sialylated compounds prior to LC-MS analysis to simplify the analyzed mixture.

2.6. Preparation of glyoxyl and APTES-GA silica supports

Sipernat 50S silica support was glyoxyl grafted as described previously [35]: Sipernat 50S silica support was dried overnight by vacuuming at 55 °C and 0.2 bar and then grafted with epoxy groups suspending 1 g of silica in 50 mL of dry toluene, 1 mL of (3-glycidyloxypropyl)trimethoxysilane (GPTMS) and 150 µL of triethylamine. After 4 hours of stirring and reflux under nitrogen, the solid was filtered off and washed thoroughly with THF. Finally, the epoxy-modified silica was dried during 12 hours at 55 °C. Afterwards, the hydrolysis of the epoxy groups was carried out by mixing 1 g of solid with 10 mL of 0.1 M H_2SO_4 for 2 hours at 85 °C. After washing with water/acetone (70:30 v/v) and drying, the resulting glyceryl-epoxide silica was subjected to a further oxidation step of the glyceryl moieties by reacting with 0.03 M $NaIO_4$ for 2 hours at room temperature. The formation of the glyoxyl groups on the silica surface was quantified by back-titration with $NaHCO_3$/KI, measuring the difference in absorbance at 405 nm of the supernatant before and after the oxidation process.

Sipernat 50S silica support was grafted with (3-aminopropyl)triethoxysilane (APTES) and glutaraldehyde (GA) as follows: 2 g of silica support was mixed with 2% (v/v) APTES solution for 2 hours at 70 °C with vigorous stirring [36,37]. After washing with MilliQ water, the support was incubated in 2.5% (v/v) glutaraldehyde solution (pH 7) for 1 hour at room temperature with moderate stirring. After washing with MilliQ water, the support was dried overnight at 35 °C and vacuum (0.2 bar).

2.7. Enzyme immobilization on glyoxyl and APTES-GA silica supports

Ultrafiltered, cell-free extracts of Tr6 and Tr15 were used for immobilization without further purification. The immobilization on the glyoxyl silica support was carried out by suspending 0.5 g of glyoxyl silica support in 5 mL of enzyme preparation diluted in 100 mM potassium bicarbonate (pH 10.5), corresponding to approximate 35 mg enzyme being offered per g support. The suspension was maintained for 2 hours at 4 °C under vigorous stirring. In order to avoid enzyme inactivation at high pH, 100 mM galactose and 25% (v/v) glycerol was added as optimized previously [31]. Subsequently, Schiff's bases of the support were reduced in 1 g/L sodium borohydride in a 160 mM phosphate-citrate buffer at optimum pH for the enzymes (pH 4.8 for Tr15, pH 5.5 for Tr6 [16]) [31,33,35]. Finally, biocatalyst was washed with 160 mM phosphate-citrate buffer at their optimum pH. The obtained biocatalysts were named glyoxyl-TrX.

Immobilization of APTES-GA silica support was carried out following the same process as above, but rather than high pH, the optimum pH (pH 4.8 for Tr15, pH 5.5 for Tr6) was used for the immobilization, which took place at 4 °C overnight; longer time was employed to compensate for the pH conditions being more favourable for enzyme stability than for immobilization. Protein concentration in the immobilization mixture before and after immobilization was determined by the BCA protein assay kit (Thermo Scientific, Rockford, USA) to estimate the immobilization efficiency. The obtained biocatalysts were named APTES-GA-TrX.

2.8. Recycling study for immobilized enzyme on glyoxyl and APTES-GA silica supports

Transsialylation activity of immobilized Tr6 and Tr15 was determined by suspending 0.5 g of glyoxyl-TrX or APTES-GA-TrX in a 50 mL reaction mixture containing 34 g/L CGMP (corresponding to 8 mM bound sialic acid) and 80 mM β-lactose in 160 mM phosphate-citrate buffer (pH 4.8 for Tr15, pH 5.5 for Tr6). Reactions were performed in duplicates following either the centrifugation method or the fed-batch method for biocatalyst recycling.

The centrifugation method was accomplished in a 100 mL round-bottom flask with stirring permitting suspension of the biocatalyst in a 50 mL reaction volume. After 50 min of reaction, all the reaction volume was centrifuged at 8600 g for 5 min at room temperature. The supernatant was kept at 90 °C for 10 min to inactivate residual activity and stored for analysis. The biocatalyst was reused by suspending it in 50 mL of fresh reaction mixture for the next reaction cycle. The whole process was repeated until 6 cycles of biocatalyst recycling were completed.

The fed-batch method was started in a 100 mL round-bottom flask with stirring permitting suspension of the biocatalyst in a 50 mL reaction volume. After 50 min of reaction, a small sample was withdrawn and centrifuged at 8600 g for 5 min at room temperature. The supernatant was kept at 90 °C for 10 min to inactivate residual activity and stored for analysis. The biocatalyst was reused by adding another 50 mL of fresh reaction mixture to a round-bottom flask already containing the reaction mixture and biocatalyst from the previous cycle. The process was repeated until 6 cycles of biocatalyst recycling were completed, adjusting the size of the round-bottom flask in order to maintain the same proportion between the reaction volume and the flask throughout.

In both methods, temperature was kept at 30 °C using a water bath placed over a heating plate equipped with a thermocouple sensor probe for temperature control. The inactivated supernatant samples were filtered on a 5 kDa polyethersulfone membrane (Vivaspin 500; Sartorius AG, Göttingen, Germany) to remove CGMP before the concentrations of SL were determined by HPAEC-PAD. For comparison, a reaction with free enzyme in a concentration corresponding to the immobilization efficiency (approximate 0.85 mg for 50 mL of reaction mixture) was monitored for 6 hours.

2.9. HPAEC-PAD analysis

Separation and quantification of sialic acid, 3'SL, 6'SL, and 3SL in the standard transsialylation activity assays were carried out by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a CarboPac[TM] PA100 (4 × 250 mm) analytical column equipped with a CarboPac[TM] PA100 (4 × 50 mm) guard column (Dionex Corp., Sunnyvale, CA) on a Dionex ICS-3000 system (Dionex Corp., Sunnyvale, CA). The operating conditions and analysis procedure have been described previously [17], and resulted in base line separation of the analytes. Sialic acid (Sigma-Aldrich, Steinheim, Germany), 3'SL, and 6'SL (Carbosynth Ltd, Compton, UK) were used as external standards. For 3SL, where no standard was available, the product formation was estimated as 3'SL equivalents. 3SL was identified previously [39], and its identity was confirmed by HPAEC-PAD and LC-MS analyses of the reaction mixtures as well as of β-galactosidase treated reaction product mixtures.

2.10. LC-MS analysis of transsialylated oligosaccharides

Identification of sialylated HMOs, GOS and XOS was performed by liquid chromatography

electrospray ionization mass spectrometry (LC-ESI-MS) on an Amazon SL iontrap (Bruker Daltonics, Bremen, Germany) coupled to an UltiMate 3000 UHPLC from Dionex (Sunnyvale, CA, USA). 5 µL sample was injected on a porous graphitized carbon column (Hypercarb PGC, 150 mm × 2.1 mm; 3 µm, Thermo Fisher Scientific, Waltham, MA, USA). The chromatography was performed at 0.3 mL/min at 70 °C on a two eluent system with eluent A (0.1% formic acid in water) and eluent B (acetonitrile). The elution profile was as follows: 0–1 min, 0% B; 1–38 min, linear gradient to 27.5% B; 38–41 min, linear gradient to 60% B; 41–75 min, isocratic 60% B; 75–80 min, isocratic 0% B. The electrospray was operated in negative mode with UltraScan mode and a scan range from 100-2000 m/z, smart parameter setting of 500 m/z, capillary voltage at 4.5 kV, end plate off-set 0.5 kV, nebulizer pressure at 3.0 bar, dry gas flow at 12.0 L/min, and dry gas temperature at 280 °C. CID fragmentation was performed using SmartFrag enhanced amplitude ramping from 80 to 120%, fragmentation time 20 ms. Identification was performed by interpretation of MS^3 spectra in Compass QuantAnalysis 2.2 (Bruker Daltonics, Bremen, Germany).

2.11. Bioinformatics

The homology model Tr16 was created with HHPred and MODELLER [40], using a crystal structure of TrSA (PDB: 1MS9) as template. Graphics were prepared with the PyMOL Molecular Graphics System, version 1.8 (Schrödinger, LLC). 1S0I was used for fitting the 3'SL ligand as well as for adjusting the rotation of the Y120 side chain in PyMOL. Average diameters of TrSA$_{5mut}$ (PDB: 1WCS) and β-galactosidase from *Aspergillus aculeatus* (PDB: 1FOB) were calculated using RasMol 2.7.5 [41].

2.12. Statistics

One-way ANOVA for determination of statistical significance was performed with JMP®, version 12.1.0 (SAS Institute Inc., Cary, NC). Statistical significance was established at $p < 0.05$.

3. Results and Discussion

3.1. Thermal stability

Thermal stability was determined for Tr15 and Tr16 (Table 2). At 30 °C, both enzymes were completely stable ($k_D = 0$), whereas their half-lives were more than 13 hours at 40 °C. At 45 °C, half-lives were around 1.5 hours, decreasing to 23 min for Tr15 and 9 min for Tr16 at 50 °C. At 55 °C, inactivation was rapid with half-lives around 3–4 min (Table 2). Comparing to the thermal stability data obtained for Tr6 and Tr13 [15,17], there is a tendency that Tr6 was more thermostable than Tr13, which was slightly more thermostable than Tr15, which was in turn slightly more stable than Tr16 (Table 2). This indicates that an increase in number of point mutations in TrSA has an increasingly negative effect on the thermal stability of the sialidase. It has been known for decades that point mutations influence protein stability differently [42]; possibly, the introduction of three adjacent, positively charged amino acids (M200K, G201K, G202K) causes charge repulsion, which is slightly harmful to the enzyme's thermal stability [43,44]. Nevertheless, all the engineered *T. rangeli* sialidases (Tr6, Tr13, Tr15, and Tr16) had a markedly higher thermal stability than TcTS [17], emphasizing the potential of the engineered sialidases in practical applications (Table 2).

Table 2. Thermal stability of Tr15 and Tr16 compared to previously obtained data on Tr6, Tr13, and TcTS. Half-life ($t_{\frac{1}{2}}$) given in min ± standard deviation with corresponding thermal inactivation constants, k_D, given in min^{-1} in parenthesis. Superscript letters indicate significant difference between the enzymes at each temperature ($p < 0.05$). Empty fields indicate that the enzyme was not tested at the given temperature.

°C	Tr6[e]	Tr13[f]	Tr15	Tr16	TcTS[f]
30		- (0)[a]	- (0)[a]	- (0)[a]	> 210 (0.003)[b]
40		> 7980 (8.7×10^{-5})[a]	> 780 (8.7×10^{-4})[a]	> 780 (8.4×10^{-4})[a]	14 ± 2 (0.051)[b]
45	- (0)[a]	236 ± 82 (0.003)[b]	106 ± 20 (0.007)[b]	84 ± 25 (0.008)[b]	1.9 ± 0.7 (0.363)[c]
50	37 ± 3 (0.019)[a]	37 ± 2 (0.019)[a]	23 ± 0.6 (0.030)[b]	9.2 ± 1.4 (0.076)[c]	0.6 ± 0.2 (1.089)[d]
55		2.8 ± 0.2 (0.251)[a]	4.3 ± 0.6 (0.161)[a]	3.0 ± 1.1 (0.229)[a]	
60	5.2 ± 0.5 (0.134)				
65	0.9 ± 0.2 (0.806)				

[e]Data from [15]; [f]data from [17].
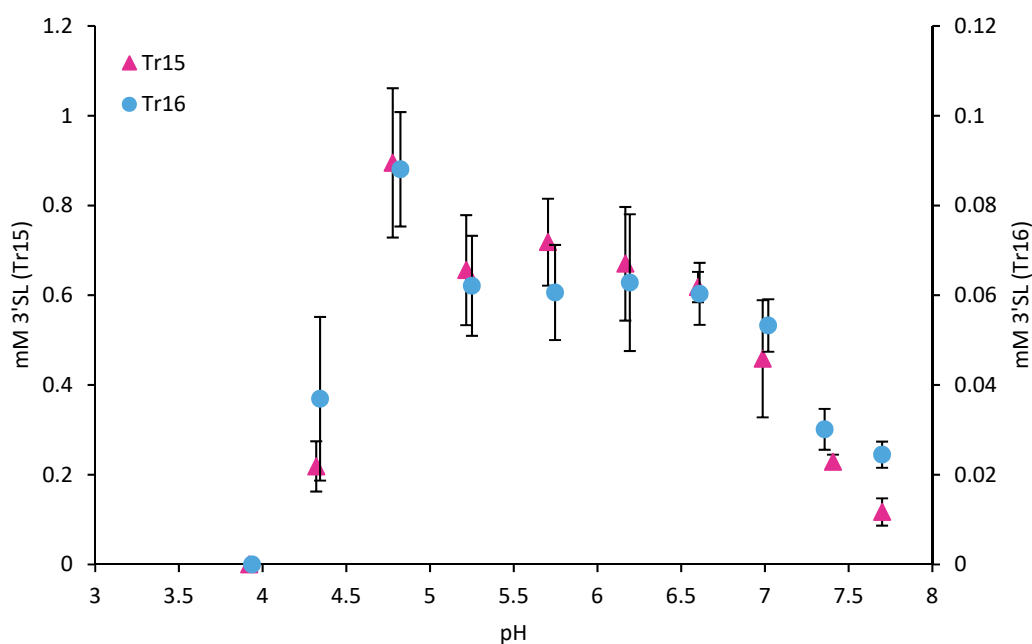
Figure 3. pH optima of Tr15 and Tr16. The effect of pH on the transsialylation activity of Tr15 (triangles; left axis) and Tr16 (circles; right axis) was measured as the concentration of 3'SL present after 10 min and 30 min of reaction, respectively. The pH was measured in the sample right after the reaction. Standard deviations of triplicate experiments are indicated.

3.2. Optimal pH

The transsialidase activity vs. pH curves appear similar for Tr15 and Tr16, both showing pH optima around pH 4.8 (Figure 3). Previously, a pH optimum around pH 5.5 was reported for Tr13 [17]. Several examples of amino acid substitutions leading to shift in pH optima have been reported. However, while some report a shift toward a more acidic pH optimum upon substituting negatively

charged amino acids with uncharged ones [45,46] as is seen here for D285G, the opposite effect has also been reported [47,48]. In any case, the introduction of T39A, F59N, and D285G led to a decrease in pH optimum from pH 5.5 to pH 4.8 for the engineered sialidases from *T. rangeli*. T39A is quite far from the 3'SL ligand (13 Å), but close to R36, which is part of the arginine triad responsible for sialyl carboxylate fixation, and the structural mutation I37L found in Tr16 (Figure 1; [49]). The other two energetic mutations are closer to the substrate (5–6 Å), and F59N is adjacent to the general acid/base catalyst D60 [49], while D285G is adjacent to the TrSA$_{5mut}$ mutation Q284P (Table 1, Figure 1). Below pH 4 there is no detectable activity (Figure 3).

3.3. Reaction rates and effect of acceptor-to-donor ratio

It has previously been shown that Tr13 has a transsialylation activity similar to that Tr6, but a significantly lower hydrolytic activity (62% on CGMP, 24% on 3'SL) [10]. In the current study, the reaction rates of the newly engineered *T. rangeli* sialidases Tr15 and Tr16 were compared to those of Tr13 (Figure 4). The transsialylation and competing hydrolysis catalyzed by Tr13, Tr15, and Tr16 were monitored for 3 hours at acceptor-to-donor ratios (A:D) of 2 and 10, using a concentration of sialic acid bound in CGMP of 8 mM and lactose concentrations of 16 mM and 80 mM, respectively. A high A:D is known to favor transsialylation [10,16,22], whereas a lower A:D is more relevant for expensive acceptors such as larger HMO core structures.

It is evident that increasing A:D from 2 to 10 had a major effect on the ratio between the transsialylation rates and the hydrolysis rate (r_T/r_H) for all three engineered sialidases (Table 3). In all cases, significantly lower hydrolysis rates were observed when A:D was increased (Figure 4). The transsialylation rates were doubled for Tr13 when A:D was increased from 2 to 10, whereas the increase was more moderate for Tr15 and absent for Tr16 (Figure 4). As a consequence, significant increases in sialyllactose (SL) yields were observed for Tr13 and Tr15 when A:D was increased from 2 to 10 (Table 3). As the reaction is a kinetically controlled synthesis, it is expected that a higher acceptor concentration (5 times higher at A:D = 10 compared to A:D = 2) would give a higher r_T/r_H (Equation 1).

Table 3. Ratio between reaction rates of transsialylation and hydrolysis ($r_T/r_H = (r_{3'SL} + r_{6'SL} + r_{3SL})/r_{SA}$), maximum concentration of SL (sum of 3'SL, 6'SL and 3SL), and regioselectivity for 3'SL in the transsialylation obtained with Tr13, Tr15, and Tr16 during 3 hours of reaction using acceptor-to-donor ratios (A:D) of 2 or 10, i.e. 8 mM bound sialic acid in CGMP and 16 or 80 mM lactose, respectively. Superscript letters indicate significant difference ($p < 0.05$) between SL levels.

	A:D	Tr13	Tr15	Tr16
r_T/r_H	2	3.8	3.7	2.8
	10	31	36	58
Max. SL produced [μM]	2	109 ± 8[c]	331 ± 85[b]	46 ± 6[c]
	10	299 ± 47[b]	690 ± 11[a]	38 ± 3[c]
Regioselectivity (% 3'SL in TS)	2	97%	98%	100%
	10	97%	97%	100%
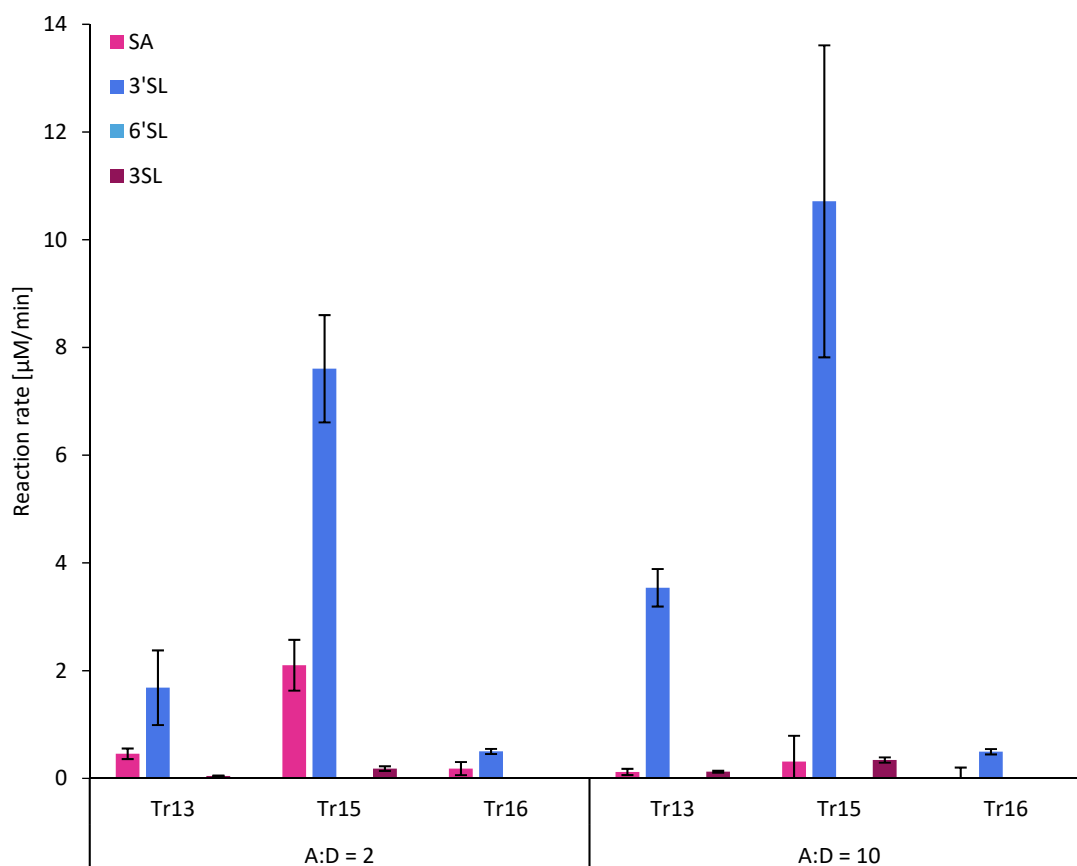
Figure 4. Initial reaction rates for production of sialic acid (SA; hydrolysis), 3'SL, 6'SL, and 3SL (transsialylation) obtained with Tr13, Tr15, and Tr16 using acceptor:donor ratios (A:D) of 2 or 10, i.e. 8 mM bound sialic acid in CGMP and 16 or 80 mM lactose, respectively. Standard deviations of duplicate experiments are indicated.

The highest r_T/r_H was obtained with Tr16 at an A:D of 10 (Table 3). However, Tr16 also had the lowest reaction rates (Figure 4) and hence the lowest transsialylation yield within the fixed reaction time (Table 3). In fact, reaction rates were so low that the large difference in r_T/r_H observed for Tr16 between the low and high A:D levels did not manifest itself in the transsialylation yields within the limited reaction time (Table 3). Indeed, it was evident from previous results [18] that the structural mutation I37L was detrimental to the reaction rate, especially in combination with the loop mutations. This trend is emphasized here: Tr15, which is the only enzyme of the three which does not harbor the structural mutation I37L (Table 1), exhibits much higher reaction rates (Figure 4) and transsialylation yields (Table 3). Indeed, the SL level achieved with Tr15 at an A:D of 2 was as high as the one achieved with Tr13 with an A:D of 10 (Table 3). In conclusion, Tr13 has benefitted from losing the structural mutation (I37L) and gaining the energetic mutations (T39A, F59N, and D285G). Previous results showed that only losing the structural mutation (i.e. TrSA$_{12mut}$) did not have a positive effect, especially not on r_T/r_H [18]. Similarly, addition of energetic mutations to Tr13 (i.e. Tr16) almost doubled r_T/r_H at an A:D of 10, but the reaction rates were significantly lower. However, at an A:D of 2, Tr16 seemed to perform more poorly than Tr13, although this difference was not significant (Table 3).

All three engineered sialidases showed high preference for producing 3'SL (Figure 4; Table 3). Tr16 produced no other detectable SLs. Tr13 and Tr15 produced small but detectable amounts of 3SL, while only Tr15 produced detectable levels of 6'SL (Figure 4). A high regioselectivity towards formation of α(2,3)-linkages was expected as this has previously been observed for the TrSA mutants as well as for TcTS [22].

Although neither Tr15 nor Tr16 outperformed their predecessor Tr13 in terms of r_T/r_H at low A:D, it is evident that Tr15 is a much more potent enzyme in terms of transsialylation rates (Figure 4; Table 3) at both high and low A:D. While Tr16 is less efficient than both Tr13 and Tr6 in terms of transsialylation rates, it is superior when it comes to the r_T/r_H ratio at high A:D, possibly evidence of a higher selectivity factor S (Equation 1). It was previously shown, that when dosed 10 times higher, Tr16 performed better than Tr15 at an A:D of 2 [18]. The high r_T/r_H ratio achieved at an A:D of 10 makes it possible to achieve high transsialylation yields without the need for tight reaction time control, which is otherwise necessary for glycosidases with a low r_T/r_H, where the transglycosylation has a transient maximum [22]. However, in most applications Tr15 appears to be the obvious choice as it is a more efficient enzyme. Indeed, it is evident from the results that a high r_T/r_H ratio is not everything: because of a markedly higher transsialylation rate, Tr15 produced much more SL at both low and high A:D even though its r_T/r_H ratios are identical to the ones obtained with Tr13 (Table 3).

3.4. Acceptor specificity of Tr15 and Tr16

The engineered *T. rangeli* sialidases, Tr6 and Tr13, as well as TcTS have been used for catalyzing the formation of a number of sialylated glycans, including GOS and HMO core structures lactose, lacto-N-tetraose (LNT), lacto-*N*-neotetraose (LnNT), lacto-*N*-fucopentaose I (LNFP I) and lacto-*N*-fucopentaose V (LNFP V) [4,6,7,10,16,17]. Indeed, the acceptor specificity seems to be broad for these enzymes, and goes beyond terminal β-galactosyl moieties as initially suggested for TcTS [5,10,22,50]. It was hypothesized that an equally broad acceptor specificity would be observed for Tr15 and Tr16.

The ability of Tr15 and Tr16 to catalyze sialylation of a broad range of relevant acceptors was tested by performing transsialylation on HMO structures LNT, LNnT, LNFP V, LNnFP V as well as on a preparation of XOS and two different preparations of GOS (570-P and Cup Oligo P). As previously observed for Tr13, both Tr15 and Tr16 could also catalyze transsialylation of the two GOS preparations, which have a varying degree of polymerization (DP) range [17] (Table 4). Transsialylation of XOS has not previously been described, but both Tr15 and Tr16 catalyzed transsialylation of low-DP XOS using CGMP as sialyl donor (Table 4). The HMO core structure LNT was sialylated by both enzymes, whereas no sialylated LNnT could be detected by LC-MS (Table 4). This was surprising, since both HMOs have previously been sialylated by Tr6 [16]. Additionally, when an α(1,3)-linked fucosyl residue was present on the reducing end terminal glucose moiety (LNnFP V), detectable transsialylation did take place (Table 4). LNFP V was also sialylated by both Tr15 and Tr16 (Table 4); this has also been reported previously for Tr6 [16]. In conclusion, the acceptor specificities of Tr15 and Tr16 are rather broad, making them useful for production of a wide range of α(2,3)-sialylated HMO and HMO-like structures. Although the major sialylated HMO in human milk appears to be 6'SL (0.6 g/L corresponding to 7% (w/w) of total HMOs for secretors and 11% (w/w) for non-secretors), appreciable levels of 3'SL (0.2–0.3 g/L) and LST a (terminally α(2,3)-sialylated LNT; 0.1-0.3 g/L) have also been identified [2], emphasizing the importance of novel routes for production of these HMOs for future addition to infant formula.

Table 4. Transsialylation of a variety of HMO structures (LNT, LNnT, LNFP V, and LNnFP V), GOS, and xylooligosaccharides (XOS) by engineered sialidases Tr6, Tr13, Tr15, and Tr16. A plus (+) indicates that transsialylation takes place, a minus (−) that no transsialylation could be detected, while no sign indicates that the compound was not tested as acceptor.

	Tr6[a]	Tr13[b]	Tr15	Tr16
LNT	+		+	+
LNnT	+		−	−
LNFP V	+		+	+
LNnFP V			+	+
XOS			+	+
GOS Cup Oligo P		+	+	+
GOS 570-P		+	+	+

[a]Data from [16]; [b]data from [17].

3.5. Immobilization of Tr6 and Tr15

As Tr15 was the most efficient of the engineered *T. rangeli* sialidases in terms of reaction rate and SL production (Figure 4; Table 3), Tr15 was selected for immobilization studies. Additionally, Tr6 was included as comparison since this engineered sialidase has previously been immobilized by a number of techniques, namely (i) His-tag immobilization on Cu^{2+}-iminodiacetic acid-functionalized magnetic nanoparticles, (ii) immobilization by physical adsorption on a regenerated cellulose membrane, and (iii) calcium alginate encapsulation of glutaraldehyde cross-linked Tr6 [15]. However, cross-linking and encapsulation were harmful to enzyme activity and rendered the enzyme inactive, whereas membrane immobilization suffered from low enzyme loading [15]. Immobilization via the His-tag on the magnetic nanoparticles improved the biocatalytic productivity, but suffered from severe enzyme leakage and/or inactivation so that only 7% of the initial activity remained after seven consecutive reaction cycles [15].

Two different methods of linking the enzyme covalently to the Sipernat 50S silica support were employed: (1) immobilization via glyoxyl groups promoted by alkaline conditions (pH 10.5), and (2) immobilization with glutaraldehyde to silica functionalized with APTES at neutral and acidic pH (Figure 5). To stabilize the enzyme at alkaline pH used in the glyoxyl method, galactose and glycerol were added to the immobilization mixture. In a preliminary study using 25% (v/v) glycerol and 100 mM galactose during incubation at 4 °C for 2 hours (corresponding to the glyoxyl immobilization time), the remaining activity was tested on CGMP and lactose using an enzyme concentration of 300 mg/L at the optimal pH of the enzyme. It was established that Tr6 and Tr15 retained 70–78% of their activity after incubation at alkaline conditions with glycerol and galactose: with Tr6 0.13 mM 3'SL was formed after incubation at pH 4.8 and 0.10 mM at pH 10.1 (corresponding to 78% remaining activity), whereas with Tr15 0.52 mM 3'SL was formed after incubation at pH 4.8 and 0.36 mM at pH 10.1 (corresponding to 70% remaining activity). To avoid such enzyme inactivation, immobilization by the APTES-GA method was carried out at the optimal pH of the enzymes (pH 4.8–5.5) although the amino group reactivity is relatively low at acidic conditions.

The immobilization efficiency was approximate 3% as determined from protein concentration of the immobilization mixture before and after immobilization (data not shown). In order to ensure successful immobilization of the engineered sialidases on the support, a large excess of enzyme was
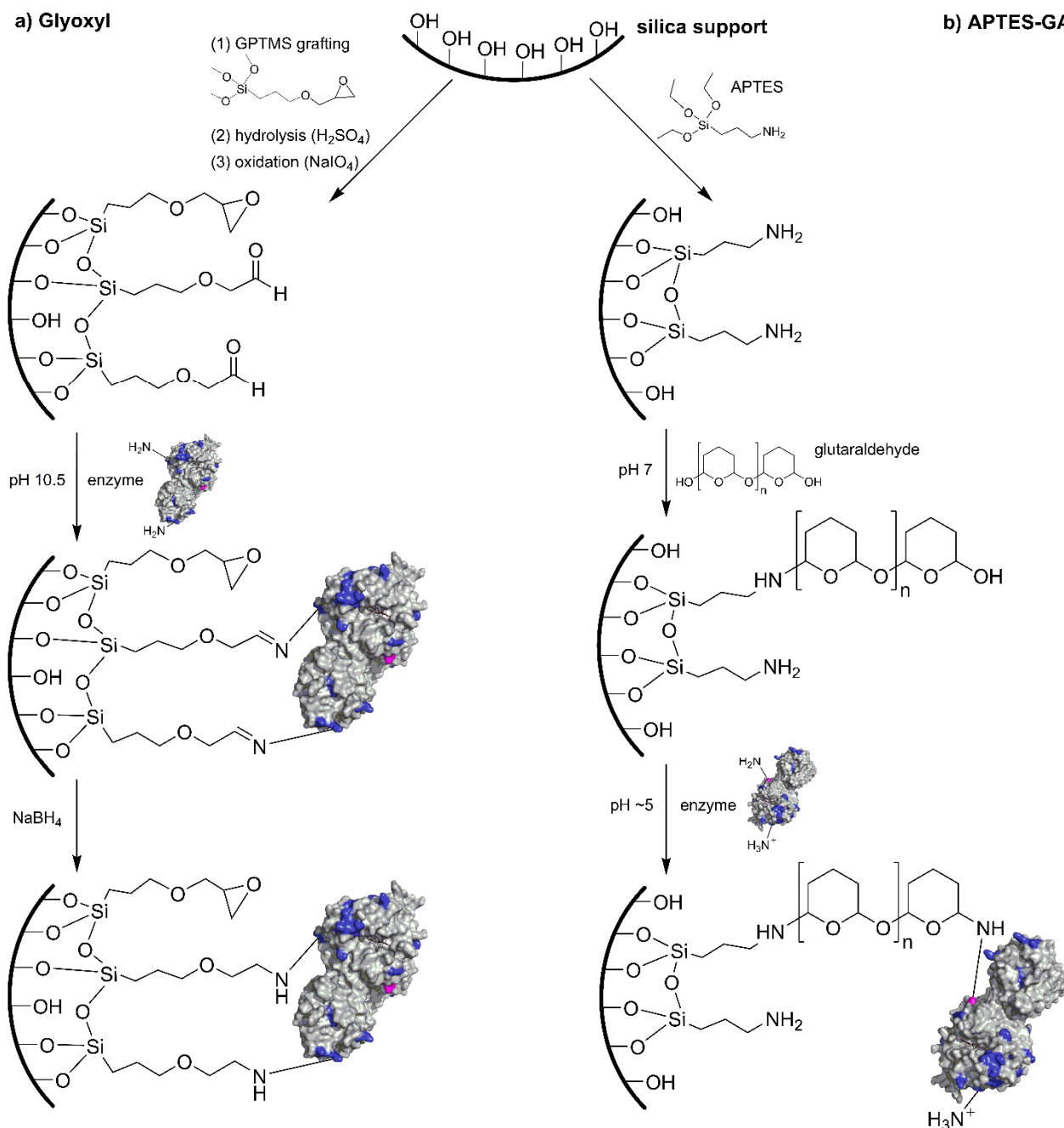
Figure 5. Covalent immobilization methods employed: a) Glyoxyl method, where the engineered sialidase is immobilized on a glyoxyl-functionalized silica support [29]; b) APTES-GA method, where the engineered sialidase is immobilized on a silica support by functionalization with APTES and glutaraldehyde (GA); the assumed conformation of glutaraldehyde at neutral and acidic conditions is based on [25,32,37]. The N-terminal is shown in pink; its amino group has a lower pK_a (7.7) than those of the Lys-side chains (10.5) [26] (shown in blue) and it is thus more likely to be uncharged at pH 4.8-5.5 and used for immobilization [23,29]. The active site is indicated by the bound 3'SL product (black).

offered to the support. As a consequence, the immobilization efficiency was low, but the amount of enzyme immobilized corresponded to an enzyme concentration of 17 mg/L in the 50 mL reaction, which was almost 3 times more than used in the previous reactions (Figure 4; Table 3). Even so, transsialylation yields were lower: a maximum 3'SL concentration of 145 μM 3'SL was achieved with 17 mg/L free Tr15 after 1 hour of reaction, which is less than one third of what was achieved with 6 mg/L free Tr15 in 1 hour (453 μM 3'SL). Probably, the increase in enzyme dosage and corresponding acceleration of primary and secondary hydrolysis caused a shift in the transient maximum to a shorter reaction time. It has previously been observed that high enzyme dosages are detrimental to transglycosylation yields [51].

In addition, regioselectivity was affected by the increased enzyme concentration. For free Tr6 (17 mg/L), regioselectivity towards formation of 3'SL decreased over the 6 hours, starting at 95% after 1 hour and decreasing to 71% after 6 hours, whereas it decreased from 90 to 82% for free Tr15. For comparison, regioselectivity was 97% for free Tr15 at 6 mg/L (Table 3). The regioselectivity was improved by immobilization: the regioselectivity towards 3'SL was complete for APTES-GA-TrX and at least 92% for the glyoxyl-Tr15 and at least 93% for glyoxyl-Tr6. In all cases, the main byproduct was 3SL. It has previously been observed, that regioselectivity of sialidases was compromised by immobilization [52,53]. However, in this case, it is evident that both enzyme dosage and immobilization affect regioselectivity.

Comparing the immobilized sialidases to their free counterparts, the remaining activity was around 50% for the glyoxyl method and around 10% for the APTES-GA method when considering the amount of 3'SL produced during the first hour of reaction. Covalent immobilization has often been found to compromise enzyme activity as it may disturb the three-dimensional structure [34,38]. Such a decrease is however often compensated by a higher enzyme stability [23]. Comparing the two covalent immobilization methods, it is evident that much higher transsialylation yields were obtained with the glyoxyl method (Figure 6). While 20–25% of the activity of TrX was lost during incubation at high pH in the glyoxyl method, this method still provided a more active enzyme than the APTES-GA method. Remaining activities between 7% and 40% compared to the free enzyme are commonly reported for the APTES-GA method [38,54,55]. When operated at acidic or neutral pH this method is expected to create initial unipoint immobilization by formation of a linkage to the N-terminal amine. In the case of TrX, the distance from the N-terminal to the active site is only approximate 26 Å and they are on the same face of the enzyme. Thus, it is conceivable that the active site is partially blocked upon APTES-GA immobilization. In contrast, the glyoxyl method which targets the amine groups of lysine, makes it possible for the immobilized enzyme to adopt more orientations, making it more likely that the active site will often face the solution.

Two different methods were employed for recycling the enzyme: (1) centrifugation, where the immobilized enzyme was removed from the reaction mixture after each 50-minute cycle and added to a new bacth or reaction mixture, or (2) fed-batch, where additional new substrate was simple added to the reaction vessel after each 50-minute reaction cycle. Obviously, the fed-batch operation could also be carried out with free enzyme, but the use of immobilized enzyme allows for subsequent recovery and use in a new fed-batch operation. The fed-batch results indicate that the immobilized sialidases were stable in the reaction mixture for at least 6 hours (Supplementary Figure S1). The difference in transsialylation yield was not significant between the two recycling methods (Figure 7), but it is evident that some activity was lost in the centrifugation method, where the biocatalyst was separated from the reaction mixture after each cycle: for the glyoxyl method, the 3'SL concentration obtained in

the sixth cycle was around 70% of that obtained in the first cycle, whereas it was only around 50% for the APTES method (Figure 6). This could either be due to leakage of the enzyme from the support or because not all of the biocatalyst is recovered in the pellet after centrifugation. In case of the former, the glyoxyl method appears to provide a stronger linkage between the enzyme and the support, as less enzyme activity is lost. This may be due to the multipoint attachment facilitated by the glyoxyl method (Figure 5) [23,29].
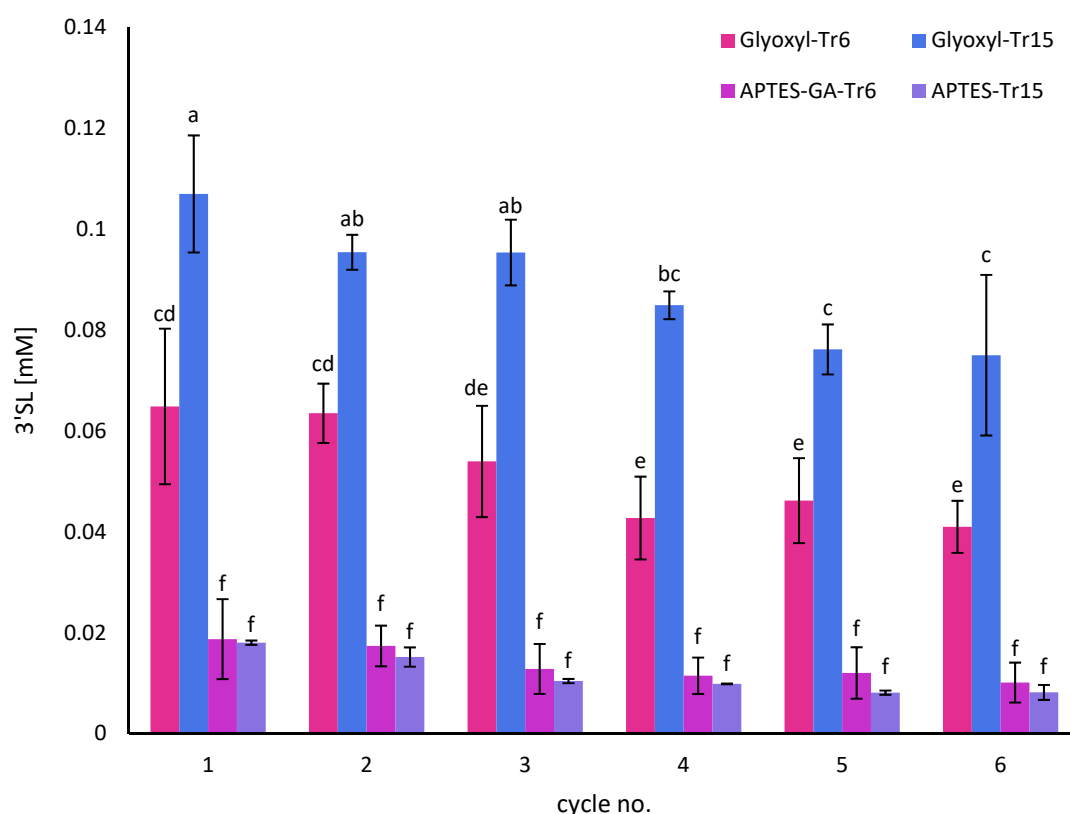

Figure 6. Recycling of immobilized Tr6 and Tr15 by centrifugation. Concentration of 3'SL obtained after immobilization of Tr6 and Tr15 on silica support by glyoxyl or APTES-GA linking. The immobilized sialidases were recycled in 6 consecutive 50-minute reaction cycles by removal of the biocatalyst from the reaction mixture by centrifugation. Reactions took place at optimal pH and 30 °C using 8 mM sialic acid bound in CGMP as sialyl donor and 80 mM lactose as acceptor. Standard deviations of duplicate experiments are indicated. Lowercase letters indicate significant difference between values ($p < 0.05$).

In terms of biocatalytic productivity (amount of product per amount of enzyme), a 1.5-fold increase was obtained for 6 cycles with the glyoxyl method compared to the free enzyme (Figure 7). Although the immobilization was fairly stable, the initial activity of the immobilized enzyme appeared decreased, giving rise to fairly low transsialylation yields and hence a lower increase in biocatalytic productivity after immobilization than expected. However, as the enzymes appeared to maintain their activity for extended reaction times in the fed-batch recycling (Figure S1), it is likely that a larger improvement in biocatalytic productivity could be obtained if the enzyme was recycled more than six

times using the glyoxyl immobilization method. For the APTES-GA method, the biocatalytic productivity was less than half of that obtained with the free enzyme, and this method is thus not recommendable for the current system (Figure 7). Previously, it was found that immobilization of Tr6 via the His-tag on Cu^{2+}-iminodiacetic acid-functionalized magnetic nano-particles increased the biocatalytic productivity 2.5 times. This method however suffered from severe enzyme leakage, to a much higher extent than observed for the covalent immobilization methods employed here (Figure 6) [15]. Instead, it was shown that using free Tr6 or Tr13 in a membrane reactor was an efficient way of recycling the engineered sialidases in the transsialylation process, since the biocatalytic productivity was increased 7 to 9 times compared to the batch reaction [15,17]. Possibly, the use of a bulky sialyl donor (CGMP) favors the use of free enzyme in these reactions. The engineered TrSA variants are structurally similar to the β-galactosidase from *Aspergillus aculeatus*, which was previously immobilized in the glyoxyl-functionalized silica support giving rise to improved transglycosylation yields [35], as they are monomeric and have a similar average diameter. Thus, immobilization of Tr6 and Tr15 in the silica support should not be hampered by mass transfer issues. However, CGMP has a pH-dependent apparent molecular mass which varies from 10–30 kDa at pH 3.5 to 20–50 kDa at pH 7.0, even though the theoretical molecular mass is only 7–10 kDa [56]. Thus, the lower transsialylation activity observed upon immobilization of Tr6 and Tr15 in the current study may be caused by mass transfer limitations for the sialyl donor.

Finally, the results obtained with the glyoxyl-immobilized sialidases corroborate that Tr15 transsialylation was improved compared to its predecessor Tr6 (Figures 6 and 7). For the APTES-GA method, the yields were too low to reveal any possible differences in performance between the two engineered sialidases (Figures 6 and 7).
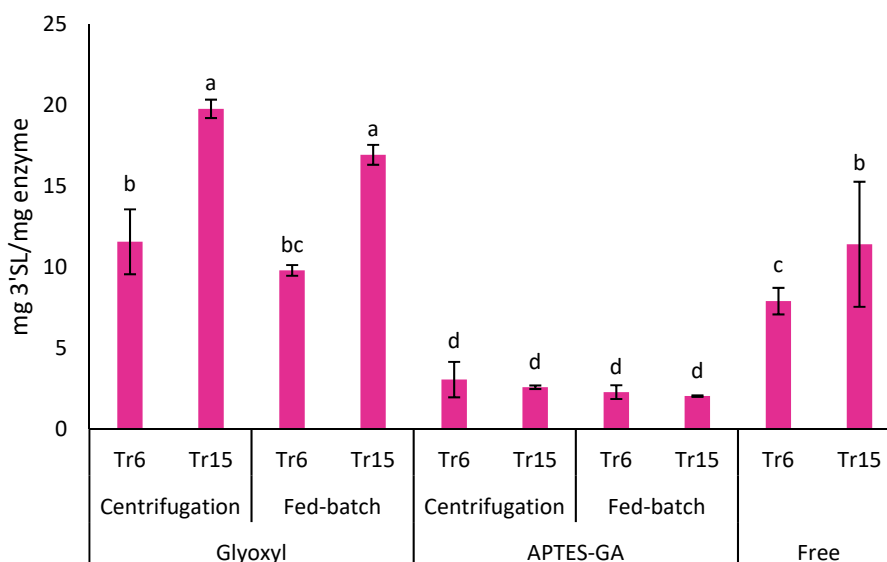
Figure 7. Biocatalytic productivity of immobilized and free Tr6 and Tr15. Given as mg 3'SL produced per mg enzyme. Standard deviations of duplicate experiments are indicated. Lowercase letters indicate significant difference between the values ($p < 0.05$).

4. Conclusions

When aiming to catalyze transglycosylation with a glycosidase, a number of parameters influence the transglycosylation performance, including transsialylation and hydrolysis rates, pH optimum, acceptor-to-donor ratio, and acceptor specificity. Of the two superior variants of the engineered sialidase from *T. rangeli*, Tr15 stood out as the most efficient enzyme for transsialylation due to higher reaction rates, although Tr16 exhibited a higher ratio between the transsialylation rate and the hydrolysis rate. Both enzymes showed broad acceptor specificity and are thus good candidates for enzymatic production of α(2,3)-sialylated glycans including HMOs. Immobilization of Tr6 and Tr15 on glyoxyl-functionalized silica led to an increase in biocatalytic productivity, even though ultrafiltered cell-free extracts were used in the immobilization process without extra enzyme purification. Furthermore, the use of glyoxyl-functionalized silica proved to be markedly better for immobilization than silica functionalized with APTES and glutaraldehyde. However, combining these results with previously obtained results on immobilization of Tr6, it is evident that immobilization of the engineered sialidases may introduce mass transfer limitations due to the use of a bulky sialyl donor (CGMP), resulting in reduced transsialylation activity.

Acknowledgements

Conflict of interest

All authors declare no conflicts of interest in this paper.

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