

Carbohydrate-Active enZyme (CAZyme) enabled glycoengineering for a sweeter future

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Abstract: One of the stumbling blocks to advance the field of glycobiology has been the difficulty in synthesis of bespoke carbohydrate-based molecules like glycopolymers (e.g., human milk oligosaccharides) and glycoconjugates (e.g., glycosylated monoclonal antibodies). Recent strides towards using engineered Carbohydrate-Active enZymes (CAZymes) like glycosyl transferases, transglycosidases, and glycosynthases for glycans synthesis has allowed production of diverse glycans. Here, we discuss enzymatic routes for glycans biosynthesis and recent advances in protein engineering strategies that enable improvement of CAZyme specificity and catalytic turnover. We focus on rational and directed evolution methods that have been developed to engineer CAZymes. Finally, we discuss how improved CAZymes have been used in recent years to remodel and synthesize glycans for biotherapeutics and biotechnology related applications.

Keywords: Glycans, Glycoconjugates, CAZymes, Glycosyl Transferases, Glycosyl Hydrolases, Transglycosidases, Glycosynthases

Highlights:

- Glycoengineering of glycans and glycoconjugates requires access to better enzymes
- Carbohydrate-Active enZymes can be improved using novel protein engineering methods
- Rational engineering utilizes CAZyme sequence information to identify mutation sites
- High-throughput screening enables advanced directed evolution methods for CAZymes

1. Introduction

Glycosylation is one of the most abundant and critical post-translational modification that takes place inside living cells where glycans (or carbohydrates) are attached covalently to other biomolecules such as proteins and lipids. Glycans are ubiquitous in nature and play an important role in regulating a plethora of biological processes such as cell-cell sensing/interaction, antibody recognition, and viral/bacterial pathogenesis (**Figure 1A**). In recent years, there has been a tremendous impetus towards production of biopharmaceuticals in the form of enzymes, monoclonal antibodies, hormones, and cytokines. Among the top ten selling pharmaceutical drugs in 2019 (**Figure 1B**), seven drugs listed were monoclonal antibodies (mAbs) that are post-translationally modified via glycosylation and are typically produced using both humanized or non-humanized mammalian cell lines [1]. The structural and compositional diversity of glycans displayed on these mAbs is often very critical for correct protein folding, thermal stability, and *in vivo* efficacy of these drugs. In fact, it has been shown that manipulating the glycosylation pattern on therapeutic proteins like mAbs, or ‘glycoengineering’, can increase drug efficacy and immunogenicity [2,3]. The glycoengineering approach to manipulate the native glycan sequence/structure provides a promising avenue to design and improve biotherapeutics particularly for time-sensitive viral diseases such as coronaviruses that are often glycosylated to evade the host immune system. Glycoengineering or glycan remodeling of protein demands simple and efficient *in vivo* or *in vitro* chemical and/or enzymatic routes for synthesis of designer

glycans. In this review article, we discuss the leading enzymatic routes for glycans biosynthesis and recent advances in protein engineering strategies that can facilitate improved enzyme specificity and efficiency. Lastly, we highlight how glycoengineering tools are critical to develop better antiviral drugs and how engineered CAZymes can address the increasing demand for designer glycans synthesis and glycan remodeling of protein based biotherapeutics.

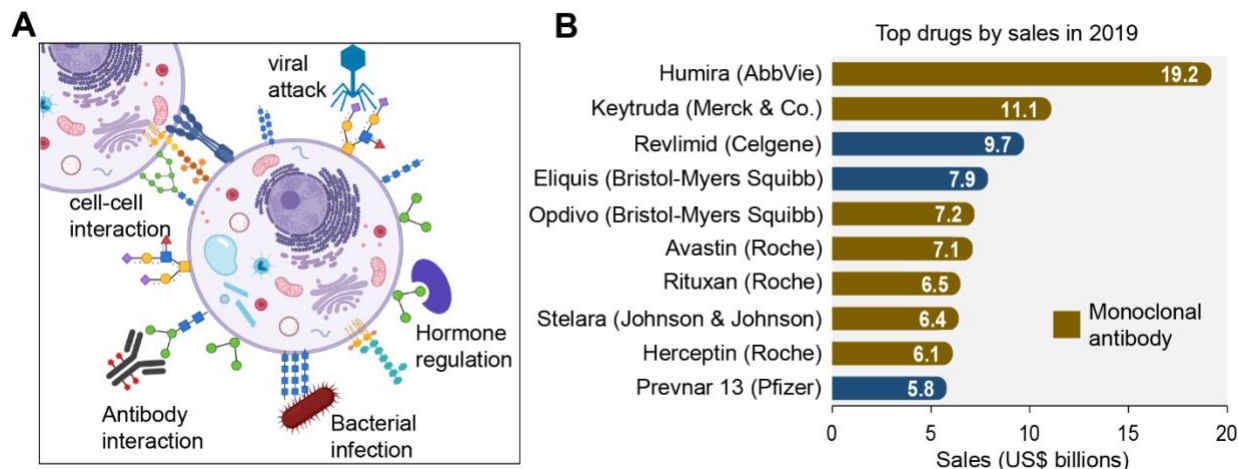


Figure 1. Glycosylation is a critical post-translational modification taking place in cells that regulates several biological processes. A) Glycans play an important role in diverse cellular processes such as cell-cell interaction, antibody and hormonal regulation, and viral/bacterial pathogenicity. B) Names of top drugs (and manufacturing companies) listed in descending order based on total global sales in 2019. Data reported is based on [1].

2. CAZymes engineering for glycans synthesis

Glycans biosynthesis is a template independent pathway that is carried out using a complex interplay of enzymes such as glycosyl transferases and glycosyl hydrolases. Glycosyltransferases (GTs) are primarily responsible for biosynthesis of most cellular glycans and glycoconjugates facilitated by transfer of nucleotide-sugar donors to either glycone or aglycone based acceptor groups [4]. On the other hand, glycosyl hydrolases (GHs) are nature's antipode of GTs that mostly catalyze hydrolysis of glycosidic linkages but can sometimes synthesize glycosidic bonds using the transglycosylation reaction mechanism. A large repertoire of these enzymes has been identified and classified into various families based on sequence and structure similarity (i.e., currently at 111 GT families and 168 GH families) into the Carbohydrate Active enZyme (CAZy) database [5]. Both GTs and GHs are further categorized based on their reaction mechanism as either inverting or retaining enzymes (**Figure 2A, 2C**). These enzymes offer exquisite regioselective and stereoselective control over traditional multi-step synthetic chemistry-based approaches. However, GTs are often membrane-bound proteins that have poor cytoplasmic expression, low solubility or stability, limited substrate specificity. Whereas, the transglycosylation pathway of GHs suffers from low yields due to subsequent product hydrolysis and the natural preference to competing water molecules as acceptor groups. Fortunately, protein engineering of GTs and GHs to address these challenges can be performed to achieve near-theoretical product yields. Over the last few years, significant advances in engineering these enzymes using both sequence/structure guided rational approaches and directed evolution based randomized approaches have been achieved as discussed in the following sections.

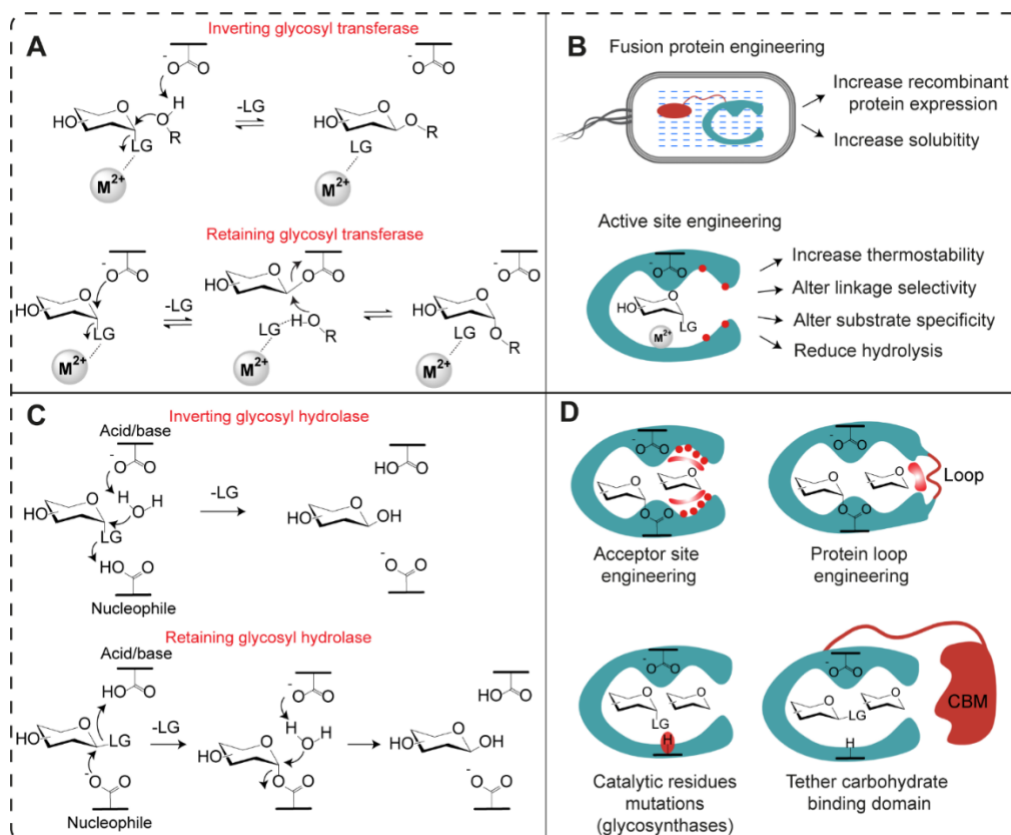


Figure 2. Rational engineering of glycosyl transferases and hydrolases. A) Mechanism of glycosyl transferases for glycosidic bond formation. B) Strategies used for engineering more efficient glycosyl transferases. C) Mechanism of β -glycosyl hydrolases for glycosidic bond hydrolysis. D) Protein engineering strategies for improving transglycosidase activity. Here, LG and CBM stands for leaving group and carbohydrate-binding module, respectively.

2.1 Sequence/structure guided rational engineering of CAZymes

More than 700,000 sequences of GTs have been deposited in the CAZy database (as of July 2020) with 2046 characterized and 273 protein databank (PDB) structures available [5]. The increasing wealth of gene sequences and protein crystal structures has greatly facilitated rational engineering of GTs to generate more efficient enzymes for glycans synthesis. Lately, the characteristic properties targeted for rational GT engineering approach extends to but not limited to (**Figure 2B**), (i) enhance heterologous expression in bacterial or yeast expression system, (ii) increase enzyme half-life, (iii) thermostability, (iv) higher specific activity, (v) minimize substrate hydrolysis, and (vi) improve or alter regio- or stereo- specificity of substrate [6]. GTs are generally membrane associated which makes it difficult to obtain soluble proteins using simple expression system such as *Escherichia coli* where most of the expressed protein ends up in inclusion bodies. Chen et al used a protein fusion strategy to improve soluble protein expression of a glucosyltransferase (UGT76G1) from *Stevia rebaudiana* [7]. A 30-phosphoadenosine-50-phosphatase (CysQ) protein was fused to the N-terminus of UGT76G1 to obtain 40% higher soluble protein expression. Enhancing glycosyl transferase expression in bacteria or yeast can enable more facile engineering to further improve GT stability and function. Li et al recombinantly expressed cyclodextrin glycosyltransferase (CGTase) in *E. coli* and subsequently improved its thermostability by investigating the effects of substituted amino acids in the active site of enzyme

[8]. In addition to achieving high active protein yields, rational modifications within glycosyl transferases are majorly focused towards substrate specificity or regioselectivity.

Multiple sequence alignments and structural analysis can provide information regarding key GT active site residues which can be probed to improve substrate recognition or modify product selectivity as reported in several recent studies [9–12]. A notable study includes switching the regioselectivity of a glucosyltransferase from *B. licheniformis* from 4'-OH to 3'-OH of resveratrol to synthesize 3-O- β -glucoside of resveratrol (polydatin) which possess improved bioavailability and pharmaceutical properties [11]. Recently, an extensive structure guided mutagenesis study of α 2,3-sialyltransferase from *Photobacterium phosphoreum* identified mutant variants with suppressed hydrolytic activity and broad substrate specificity [13]. Rational engineering approach guided by detailed sequence and structural analysis performed in these listed examples provide future several directions to engineer other glycosyl transferases available currently in the database with available PDB or highly homologous structures.

As opposed to glycosyl transferases, glycosyl hydrolases have evolved to mostly facilitate hydrolysis of high molecular weight carbohydrates to produce simple soluble sugars by attack of nucleophilic water towards the anomeric glycosidic bond carbon centers. Nonetheless, several GH enzymes can synthesize glycans via the transglycosylation mechanism by replacing the nucleophilic water with a suitable glycone or aglycone acceptor group as seen in nature [14]. Unfortunately, transglycosylation reactions suffer from low yields due to subsequent hydrolysis of synthesized products using the endogenous GH nucleophile. To circumvent this limitation, reaction conditions and substrate concentrations are often varied to shift the equilibrium towards transglycosylation [15]. Usvalmapi et al [16] altered the reaction equilibrium of GH29 α -fucosidase derived from *Aspergillus niger* by incubating with high concentrations of lactose and fucose to synthesize 1-fucosyllactose as the major transfucosylation product. Although this approach did not require use of any activated sugars, decreasing substrate concentrations with prolonged reaction times and intrinsic hydrolysis activity of native enzyme results in poor transglycosylation efficiency. Therefore, utilizing classical reaction engineering methods alone to improve transglycosylation yield can prove to be inefficient and is therefore coupled with protein engineering methods to design more efficient transglycosidases. Over the past few years different protein engineering strategies guided by protein sequence and structure have been employed to improve the transglycosylation/hydrolysis (T/H) ratio of mutant or engineered transglycosidases which are summarized briefly here (**Figure 2D**); (i) active site non-catalytic residue mutations, (ii) loop engineering near active site, (iii) catalytic residues (nucleophile and acid/base) mutation, and (iv) appending carbohydrate binding domains to catalytic domain. The transglycosylation efficiency is highly dependent on the enzyme structure to associate with non-water acceptor groups. For CAZymes, with solved structures or reliable homology models, active site modifications can be easily predicted using substrate docking and molecular dynamics simulations. Amino acid mutations that increases acceptor sugar group binding interactions can be identified to facilitate the acceptor attack of the enzyme-substrate complex. Additionally, predictions based on sequence similarity to existing homologous transglycosidases would narrow the search space for computational tools. Lundemo et al observed that mutating an asparagine residue (N220) in the acceptor site of GH1 β -glucosidase from *Thermotoga neapolitana* to more hydrophobic residues (N220F, N220W) resulted in upto 8-fold increase in the T/H ratio [17]. The asparagine residue in the wild type enzyme promoted water mediated interactions. Therefore, making the site more hydrophobic for mutant enzymes facilitated deglycosylation using acceptor sugar hydroxyl group attack. Likewise, the active site residue (E361) of GH 42 β -galactosidase

was mutated by Strazzulli et al to a smaller and less hydrophilic glycine residue to increase the transglycosylation efficiency by 177-fold [18]. In another study, Tran et al noticed that the hydrolytic activity of another GH1 transglucosidase (Os9BGlu31) enzyme was increased when Leu241 was mutated to a hydrophilic aspartic acid [19]. The observations made by Lundemo et al, Strazzulli et al and Tran et al suggests a direct relationship between the active site hydrophobicity and enzyme transglycosylation efficiency. However, the complex architecture of the enzyme active site refutes this overly simplistic prediction as seen by Tran et al where a more hydrophobic residue (W243) was replaced with a hydrophilic asparagine residue (W243N) to improve the transglycosylation efficiency of Os9BGlu31. Surprisingly, while the L241D mutation had initially increased hydrolysis activity of Os9BGlu31, this mutation also increased the transglycosylation rate when coupled with W243N by creating a positive epistasis. These non-linear effects of mutations on engineered enzyme activity makes it difficult for the researchers to use classical rational methods alone to engineer enzymes.

Nevertheless, mutations that shield the substrate-binding pocket and provide favorable interactions for acceptor/donor sugar binding are often good starting targets for engineering more efficient transglycosidases. A recent study comparing two enzymes of cycloalternan (CA) metabolic pathway in a foodborne pathogen *Listeria monocytogenes* identified key parameters that confer transferase versus hydrolase activity to these enzymes [20]. The CA forming enzyme (*LmCAFE*) and CA degrading enzyme (*LmCADE*) use similar catalytic apparatus to catalyze either synthesis or hydrolysis of the $\alpha(1,3)$ glucan linkages, respectively. Comparative analysis of these two enzymes revealed distinct structural features such as conformational changes in the loop near the active site and a non-catalytic loop domain that promoted acceptor sugar binding in the *LmCAFE* enzyme active site. The active site loop containing a hydrophobic tryptophan residue (W430) assumes a deeper conformation in *LmCAFE* enzyme to shield the active site and allowed for CH- π substrate stacking. Whereas a shallow conformation of the loop in *LmCADE* promotes hydrolysis. A similar loop engineering approach was used by Jamek et al to improve the activity of β -N-Acetylhexosaminidase (HEX1) to synthesize lacto-N-triose II from lactose and chitobiose [21]. Sequence alignment of closely related GH20 family enzymes revealed key loop residues that were introduced in HEX1 enzyme to provide a 9-fold increase in transglycosylation activity. The work by Light et al also noted the presence of carbohydrate binding domain (CBM) in *LmCAFE* that provides additional non-catalytic interactions that orients and increases the effective substrate concentration near the catalytic domain for efficient transferase activity. Recently, Bandi et al also showed that tethering a CBM domain (CBM3a) to an inactive GH5 cellulase mutant (CelE-E316G) restored its transglycosylation activity and minor hydrolytic activity toward activated soluble donor sugars like p-nitrophenyl cellobiosides [22]. It was inferred that the hydrophobic cleft of CBM3a provided additional substrate interactions that participates in either a non-catalytic S_N2 mechanism or a catalytic S_{Ni} -like mechanism to facilitate synthesis of cello-oligosaccharides. The transglycosylation activity of CBM3a linked CelE-316G was found to be >140 fold higher than just the control CelE-E316G domain. Both these studies establish a promising approach of appending catalytic domains with a suitable non-catalytic binding protein loop/domain that facilitate the glycosyl transfer step to develop more efficient transglycosidases.

The presence of intact catalytic nucleophile residue hinders the transglycosylation efficiencies of engineered enzymes as the synthesized products are prone to subsequent hydrolysis, especially during longer reaction times. Employing classical reaction engineering methods to optimize biosynthesis conditions such as pH, temperature, and solvent composition can partly address these limitations. Along with engineering the active site of GH1 glucosidase, Lundemo et al [17]

eliminated mutant hydrolytic activity without affecting transglycosylation activity by using high pH reaction conditions. Alternatively, since the early 2000s, a subset of engineered transglycosidases called glycosynthases have been designed by mutating the catalytic nucleophile of glycosyl hydrolase to a smaller non-nucleophilic residue such as alanine, serine, or glycine [23]. These mutant enzymes catalyze the glycosidic bond formation using modified activated donor sugars which structurally resembles the enzyme-donor sugar transition state intermediate. The resultant products are not hydrolyzed further as these glycosynthase enzymes lack a catalytic nucleophile residue to initiate the hydrolysis reaction step. This approach has enabled engineering multiple GH family enzymes involving both retaining and inverting mechanisms to synthesize several oligosaccharides and polysaccharides [23,24]. Of late, chitinases from GH 18 [25–27] and α -galactosidases from GH 97 [28] were successfully engineered to glycosynthases by mutating their catalytic residues. Apart from the nucleophilic residues, the catalytic acid/base residues were subjected for mutagenesis to generate thioglycoligases that can synthesize thiol-containing glycoconjugates [29]. Here, catalytic acid/base mutant (E314A) of *Streptomyces plicatus* GH20 hexosaminidase utilized GlcNAc and GalNAc donors and coupled them to thio-containing acceptor groups. The general base is mutated to alanine to prevent the deprotonation of water molecule during the de-glycosylation step while allowing attack by a low pKa thio-group of the acceptor sugar. Combining the catalytic residue mutations with other active site or loop engineering approaches can be very powerful in rendering highly efficient transglycosidases, particularly with increased availability of solved glycosyl hydrolase structures.

2.2 Selective screening strategies guided randomized engineering of CAZymes

There are currently a vast majority of glycosyl hydrolases and transferases that are yet to be structurally or functionally characterized which has impeded the use of above-mentioned rational approaches for engineering CAZymes. Directed evolution is an alternative strategy where the parent enzyme template sequence is iteratively mutated and rapidly screened until a mutant with desired functionality is identified [30]. The crucial process in the directed evolution methodology is the screening strategy that is applied to identify the desired improved constructs from a pool of redundant or inactive variants. The degree of screening strategies ranges from low-throughput to ultra-high throughput which depends on the detection principle, sensitivity, and instrumentation. The screening strategies developed for glycosyl hydrolase or glycosyl transferase can be broadly classified into two types; (i) products based, and (ii) by-product (or donor leaving group) based. The products-based screening methodology relies on chemically tagging the donor and/or acceptor sugar with a fluorophore or suitable tag that would have a distinct physicochemical function upon glycoside product formation. The resultant product with the label can act as a trigger for transcription of a reporter gene inside the cell [31] or the label molecule can be cleaved using an enzyme specific to the formed product after which the cleaved label is detected using absorbance, colorimetric, or fluorescence measurements. Also, both the donor and acceptor molecules can be tagged with a fluorescent molecule each which show FRET (Fluorescence resonance energy transfer) like behavior upon formation of the glycosidic linkage [32]. Examples of studies where fluorescent substrates were used for directed evolution of glycan synthesizing enzymes include Aharoni et al [33], Yang et al [34], Mayer et al [35], Kim et al [36] and Shim et al [37]. A similar strategy of employing fluorescent substrates was recently used by Tan et al [38] to identify α 1,3-fucosyltransferase mutants that showed 6-fold and 14-fold improved activity for Lewis x and 3'-fucosyllactose synthesis, respectively. The reaction between GDP-fucose donor sugars and fluorescent acceptor sugar groups results in a fluorescent fucoside product that is entrapped inside *E. coli* cells and can be further isolated using fluorescence activated cell sorting

(FACS) instrument. Armstrong et al [39] used fluorogenic and chromogenic substrates to develop a screening strategy for identifying donor specificity and acceptor specificity for glycosynthase enzymes. These substrate-based methods are very specific to the product of interest but are highly efficient and selective in identifying improved mutants.

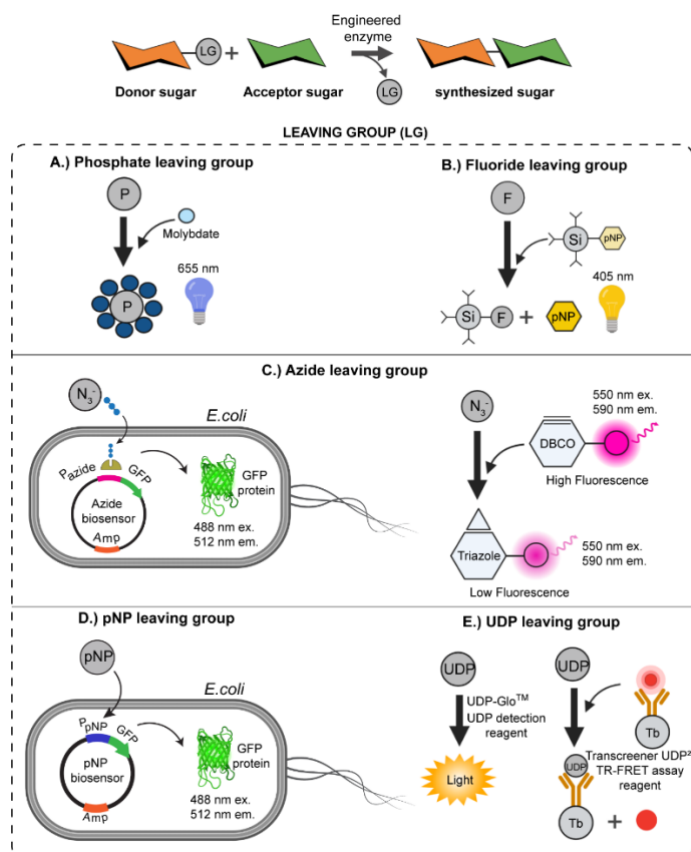


Figure 3. Five leading examples of leaving group (i.e., phosphate, fluoride, azide, pNP, and UDP/GDP) based low to high-throughput based screening strategies to enable directed evolution of glycan synthesizing enzymes.

Alternatively, screening of glycosynthases have been done in the past by detecting the by-products of the glycosylation reaction [40]. When glycosyl fluoride is used as a donor sugar for many glycosynthase reaction it releases a fluoride anion which decreases the pH of the reaction mixture. Therefore, pH indicators such as bromocresol purple, methyl red, and bromophenol blue were used to measure the reduction in cellular pH to quantify the intracellular expressed glycosynthase activity. Similar strategies have been developed for other types of leaving groups used in glycosyl transferase and modified glycosidase reactions. The most common leaving groups used for these reactions include the phosphate, fluoride, azide, pNP, and UDP/GDP groups where each group has an exclusive detection strategy developed (**Figure 3**). The phosphate ion complexes with molybdate to form molybdenum blue that has strong absorbance at 655 nm [41]. A low-throughput 96-well plate assay was developed by Macdonald et al [41] to identify new glycoside phosphorylases from GH 94 and GH 149 by monitoring the release of inorganic phosphate based on the formation of molybdenum blue. For detection method of fluoride, two new chemosensor assays consisting of silyl ether of fluorogenic methylumbelliferone or chromogenic p-nitrophenol were used for engineering *Bacillus licheniformis* 1,3-1,4-β-

glucanase [42] and β -glucosynthases from *Rhizobium radiobacter* and *Micrococcus antarcticus* [43].

Likewise, to detect azide ion released when using glycosyl azides as donor sugars, two recent strategies involving azide-specific promoter based reporter green fluorescent protein (GFP) expression [44] and click chemistry based fluorescent quenching [45] have now been developed. The former method involves a modified synthetic promoter which transcribes a GFP protein only in the presence of azide ion but not with glycosyl azide. In the latter method, the click chemistry reaction product formed with inorganic azide and fluorescent cyclooctyne was found to have lower fluorescence than the click reaction triazole product of glycosyl azides and fluorescent cyclooctyne that ultimately forms the basis for isolation of improved mutants. Similar to the azide based promoter system, p-nitrophenol (pNP) can now be also detected using a synthetic promoter that was originally developed for organophosphate hydrolase enzyme [46], but could be readily used for transglycosidase/glycosynthase screening as well. Finally, the UDP group which is one of the most common leaving group for glycosyl transferase reaction can be detected using commercially available UDP detection assays such as UDP-Glo™ reagent [47] and Transcreener® UDP² TR-FRET Assay [48]. The leaving groups-based detection strategy are not substrate or enzyme specific and hence could be used applied universally for all families of glycosyl hydrolases and glycosyl transferases. However, these techniques should be supported with additional product characterization for glycan synthesis since both hydrolysis and glycosylation can also lead to the formation of detected by-products. In summary, both screening methods have their respective pros-cons and should be used based on the enzyme template used for engineering.

3. The need for glycoengineering to provide better therapeutic solutions is NOW!

The demand for glycoengineering has rapidly emerged in recent years, particularly because viral pathogens utilize cell surface glycoproteins to mediate pathogenesis [49]. The recent COVID-19 pandemic caused by betacoronavirus SARS-CoV-2 emphasizes this issue as the glycans on spike protein (S-protein) shield the virus from neutralizing antibodies. Therefore, therapeutic development efforts have been targeted towards glycoengineering these S-protein glycan shields to present α -gal epitopes that can improve anti-viral immune response [50]. Also, several glycosylated antibodies with diagnostic and neutralizing properties that are specific to SARS-CoV-2 are now in the development phase. The precise glycan pattern of these engineered antigens and antibodies are critical for their efficacious function and overall safety.

Engineered cellular hosts such as mammalian, yeast, insect, and bacterial cells that recombinantly express glycosylating enzymes are being used to control the heterogeneity of glycan patterns [51]. Alternatively, *in vitro* techniques using engineered transglycosidases and glycosyltransferases have also provided a promising avenue to amend the native glycosylation patterns to produce the desired glycoforms [52,53]. Other glycan-centric biotherapeutic approaches include the use of polysaccharides such as glycosaminoglycans and marine polysaccharides and their derivatives [54]. Commercial biomanufacturing of natural and artificial polysaccharides glycoforms for glycan remodeling and designer glycoproteins production using industrially relevant hosts such as *E. coli*, Chinese Hamster Ovary (CHO), and yeast cells still requires further glycoengineering improvements. Currently, *E. coli* cannot natively produce glycoproteins as it lacks glycosylation machinery while yeast and insect cells could produce immunogenic glycan patterns. Although some mammalian cells can produce human like or 'humanized' complex glycans, a significant heterogeneity in glycoform patterns is observed with

subtle changes in culture conditions or cell line type. Distinct glycoengineering strategies are currently being used to incorporate glycosylation genes into *E. coli* cells [55] or corresponding cell free systems [56], knock out of immunogenic genes in yeast or insect cells [57,58], and cloning engineered genes into mammalian cells [59] to generate designer glycans or glycoconjugates synthesizing cellular factories.

4. Conclusion

Owing to the critical biological roles of glycans in biology there has been a tremendous interest in development of methods for glycans biosynthesis. Recent advancements in protein engineering have enabled scientists to design more efficient enzymes to synthesize glycans such as human milk oligosaccharides, chitin oligosaccharides, N-linked glycans, O-linked mannose and sialylated globo-series glycans which all hold tremendous commercial interest. However, a large repertoire of efficient enzymes is still required to meet growing research and commercial needs. Incorporating advanced computational modeling (e.g., machine learning) with directed evolution assays derived large enzyme structure-function datasets can rapidly enable development of our glycoengineering toolkit to create cellular factories that can synthesize bespoke complex glycans products starting from simple sugar substrates.

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1. Light, S. H. *et al.* Transferase Versus Hydrolase: The Role of Conformational Flexibility in Reaction Specificity. *Structure* **25**, 295–304 (2017).

Summary: Comparative analysis of two enzymes of *Listeria monocytogenes* that use same catalytic apparatus but differ in functional preference to either hydrolysis or transglycosylation was performed. The analysis reveal key structural differences such as loop dynamics and presence of non-catalytic domains that contribute for transglycosylation.

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Summary: Binding site residues were subjected to in-silico mutagenesis and computationally analyzed for their interaction with donor and acceptor sugars. Predicted effect on enzyme specificity and activity were tested using experimental analysis.

3. Macdonald SS, Armstrong Z, Morgan-Lang C, Osowiecka M, Robinson K, Hallam SJ, Withers SG: **Development and Application of a High-Throughput Functional Metagenomic Screen for Glycoside Phosphorylases.** *Cell Chem Biol* 2019, **26**:1001-1012.e5.

Summary: A high throughput functional screenig method for glycoside phosphorylases was developed based on molybdenum blue formation. This method was versatile in identifying phosphorylases from six different families of glycosyl hydrolases with different substrate specificity.

4. Shivatare SS, Huang LY, Zeng YF, Liao JY, You TH, Wang SY, Cheng T, Chiu CW, Chao P, Chen LT, et al.: **Development of glycosynthases with broad glycan specificity for the efficient glyco-remodeling of antibodies.** *Chem Commun* 2018, **54**:6161–6164.

Summary: The glycosynthase activity of an GH 18 endoglycosidase was enhanced by systematic mutation of active site residues. The generated glycosynthase mutants showed excellent transglycosylation activity towards broad range of high mannose, hybrid and complex types of N-glycans.

5. Li T, Tong X, Yang Q, Giddens JP, Wang LX: **Glycosynthase mutants of endoglycosidase S2 show potent transglycosylation activity and remarkably relaxed substrate specificity for antibody glycosylation remodeling.** *J Biol Chem* 2016, **291**:16508–16518.

Summary: A model glycosynthase enzyme was engineered to transfer complex, high-mannose, and hybrid type of N-glycans for antibody glycosylation remodeling. The glycosylation pattern on two therapeutic monoclonal antibodies were remodeled using the engineered glycosynthase enzyme.

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Summary: The impact of non-catalytic carbohydrate binding domain (CBM3a) on transglycosylation mechanism and activity of GH5 cellulase was tested. It was observed that CBM3a was able to recover and directly participate in transglycosylation reaction of inactive enzyme.

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Summary: The active site motifs of a newly discovered C-glycosyltransferase was studied. This information was used to further generate a pool of variants with diverse acceptor and donor specificities.

3. Tan Y, Zhang Y, Han Y, Liu H, Chen H, Ma F, Withers SG, Feng Y, Yang G: **Directed evolution of an α 1,3-fucosyltransferase using a single-cell ultrahigh-throughput screening method**. *Sci Adv* 2019, **5**:eaaw8451.

Summary: An α 1,3-fucosyltransferase enzyme subjected to random mutagenesis and reacted with fluorescently labelled substrates. The subsequent fluorescent oligosaccharide products of fucosylation reaction are retained in the cells which was used as a basis for screening enzymes with 6-fold and 14-fold increase activity for synthesizing Lewis x and 3'-fucosyllactose.

4. Tegl G, Hanson J, Chen HM, Kwan DH, Santana AG, Withers SG: **Facile Formation of β -thioGlcNAc Linkages to Thiol-Containing Sugars, Peptides, and Proteins using a Mutant GH20 Hexosaminidase**. *Angew Chemie - Int Ed* 2019, **58**:1632–1637.

Summary: The catalytic acid/base of GH20 exo-hexosaminidase was mutated to alanine to create a mutant enzyme that can catalyze thioglycosylation. The resultant mutant had broad acceptor specificity to thio-containing sugars, peptides and proteins.

5. Armstrong Z, Liu F, Chen HM, Hallam SJ, Withers SG: **Systematic Screening of Synthetic Gene-Encoded Enzymes for Synthesis of Modified Glycosides**. *ACS Catal* 2019, **9**:3219–3227.

Summary: An high throughput screening method was used to evaluate the donor and acceptor species using with glycosyl hydrolases which were then used with corresponding glycosynthases for catalyzing glycosylation reaction.

Table of Contents Graphic or Graphical Abstract

