Bacterial glycobiotechnology: A biosynthetic route for the production of biopharmaceutical glycans

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Abstract

The recent advancement in the human glycome and progress in the development of an inclusive network of glycosylation pathways allow the incorporation of suitable machinery for protein modification in non-natural hosts and explore novel opportunities for constructing next-generation tailored glycans and glycoconjugates. Fortunately, the emerging field of bacterial metabolic engineering has enabled the production of tailored biopolymers by harnessing living microbial factories (prokaryotes) as whole-cell biocatalysts. Microbial catalysts offer sophisticated means to develop a variety of valuable polysaccharides in bulk quantities for practical clinical applications. Glycans production through this technique is highly efficient and cost-effective, as it does not involve expensive initial materials. Metabolic glycoengineering primarily focuses on utilizing small metabolite molecules to alter biosynthetic pathways, optimization of cellular processes for glycan and glycoconjugate production, characteristic to a specific organism to produce interest tailored glycans in microbes, using preferably cheap and simple substrate. However, metabolic engineering faces one of the unique challenges, such as the need for an enzyme to catalyze desired substrate conversion when natural native substrates are already present. So, in metabolic engineering, such challenges are evaluated, and different strategies have been developed to overcome them. The generation of glycans and glycoconjugates via metabolic intermediate pathways can still be supported by glycol modeling achieved through metabolic engineering. It is evident that modern glycans engineering requires adoption of improved strain engineering strategies for creating competent glycoprotein expression platforms in bacterial hosts, in the future. These strategies include logically designing and introducing orthogonal glycosylation pathways, identifying metabolic engineering targets at the genome level, and strategically improving pathway performance (for example, through genetic modification of pathway enzymes). Here, we highlight current strategies, applications, and recent progress in metabolic engineering for producing high-value tailored glycans and their applications in biotherapeutics and diagnostics.

Keywords: bacteria; pathway engineering; engineered glycans; glycoconjugates; biopharmaceutical uses

Abbreviations

ABC, ATP-binding cassette; **Ac4GlcNAz**, tetraacetyl N-azidoacetylglucosamine; **α-(1,3)-FucT**, alpha-(1,3)-fucosyltransferase; **Az-Pse**, azido-pseudaminic acid; **β-(1,4)-GalT**, Beta-1,4-galactosyltransferase; **Car**, carbapenems; **CL**, cordiolipin; **CPC**, cephalosporin C; **CPSs**, capsular polysaccharides; **CS**, chondroitin sulphates; **CuAAC**, copper-catalysed azide-alkyne cycloaddition; **DNP**, 2,4-dinitrophenol; **ENGases**, β-N-acetylglucosaminidases; **EPS**, exopolysaccharides; **ER**, endoplasmic reticulum; **FACS**, fluorescence-activated cell sorting; **F-6-P**, fructose-6-phosphate; **GAGs**, glycosaminoglycans; **GalNAc**, N-acetylgalactosamine; **geOMVs**, glycoengineered OMVs; **Glc3Man9GlcNAc2**, triglucosylated high-mannose-type tetradecasaccharide; **GlcNAcβ**, N-acetylglucosamine β; **GlcNAz-CNP**, 2-chloro-4-nitrophenyl-N-azidoacetylglucosamine; **GTases**, glycosyltransferases; **HA**, hyaluronic acids; **HAS**, hyaluronic acid synthase; **HMOs**, human milk oligosaccharides; **HS**, heparin/heparin sulphates; **HUS**, hemolytic-uremic syndrome; **IcL**, isocitratelyase; **KDO**, 3-deoxy-d-mannooctulosonic acid; **KS**, keratin sulphates; **Leg**, legionaminic acid; **LLO**, lipid-linked oligosaccharide; **LNT II**, lacto-N-triose II; **LPS**, lipopolysaccharides; **Man3GlcNAc2**, mannose3-N-acetylglucosamine2; **MetK1-sp**, S-adenosylmethionine synthetase; **MGE**, mobile genome expression; **MOE**, metabolic oligosaccharide engineering; **MurNAc**, N-acetylmuramic acid; **MurU**, MurNAc α-1 phosphate uridylyltransferase; **NADPH**, nicotinamide adenine dinucleotide phosphate; **NDP**, nucleoside diphosphate; **NeuAc**, N-acetyl-d-neuraminic acid; **NMP**, nucleoside monophosphate; **OMVs**, outer membrane vesicles; **OSTase**, oligosaccharyltransferase; **OSTs**, oligosaccharyltransferase; **OTase**, oligosaccharyltransferase; **PCA**, phenazine-1-carboxylic acid; **PCN**, phenazine-1-carboxamide; **Pse**, pseudaminic acid; **SAM**, S-adenosylmethionine; **STEC**, shiga toxin-producing *E. coli*; **Tpase**, transpeptidase; **UDP-GlcNAc**, uridinediphosphate N-acetylglucosamine; **Und-PP**, undecaprenyl pyrophosphate; **UTP**, uridine-5′-triphosphate; **XPDH**, xilitol-5-phosphate dehydrogenase

1. Introduction

The availability of comprehensive information on many biological pathways across phylogeny is a breakthrough in biochemistry. In order to create a glycoconjugate, a carbohydrate, also known as a donor sugar (monosaccharide subunit), is joined with another molecule hydroxyl or other functional groups by the process of glycosylation (Helenius and Aebi, 2001). Glycolysation is a key post-translational modification of proteins that affects the natural functions of the protein including its physicochemical characteristics, localization, molecular interactions, and half-life (Strutton et al., 2019). In cells, five different types of glycosylation take place that are not specific to a single protein. For instance, (i) glycans can attach to the nitrogen of asparagine or arginine side chains (N-linked); (ii) the hydroxyl group of tyrosine, threonine, serine, and hydroxyproline side chains (O-linked); (iii) the phosphate group of a phosphoserine (P-linked); (iv) the carbon on a tryptophan side chain (C-linked); and (v) the components of the glycophosphatidylinositol (Schjoldager et al., 2020). Among them, N- and O-linked glycosylations are associated with several therapeutic glycoproteins (Reily et al., 2019). With the advance in modern genetic engineering tools & techniques, the cell's glycosylation pathways can be used to create a wide range of modified glycans. This process has been successfully displayed in a variety of cell types, such as bacteria, fungi, plants, and mammalian cells (Varki, 2017).

Glycoengineering is the technique of modifying glycans to make them recombinant, medically effective, and safe for human intake without inducing immune reactions (Ma et al., 2020). By changing the cellular glycosylation process in various ways, changed glycoproteins are formed during glycosylation, producing engineered glycans with modified size, charge, and solubility. Improved therapeutic effects and involvement in receptor growth and ligand formation are two benefits of tailored glycans (Bailey, 1991). Engineering-wise, the capacity to control and re-design the various glycan structures at the site of synthesis on cell surfaces provides a potent way to understand and alter the molecular processes underlying glycan-mediated signaling events (Stanley et al., 2022). This results in a systematic alteration of glycans during subsequent cellular consequences. However, a thorough understanding of the genes in charge of producing the glycosyltransferase enzyme and the substrates necessary for synthesizing the matching glycans is necessary for successful glycoengineering. Glycan biosynthesis enzymes, such as hydrolases and glycosyltransferases, are produced by a number of genes recently been discovered in past decades. Modern genetic engineering methods simply provide a variety of customized glycans by altering these crucial gene sequences (Hossain et al., 2016).

Metabolic engineering techniques have been widely used, among other approaches, to boost the production of metabolites through genetic engineering. To harness such biological processes for energy transmission, chemical transformation, and supramolecular assembly products, targeted metabolic pathways including all sets of enzymes catalyzing the glycan synthesis process in an organism should be purposely altered via metabolic engineering (Bailey, 1991). By modifying desirable biochemical processes or changing individual genes, either by introducing new genes or deleting particular regions through recombinant DNA technology, metabolic engineering focuses on the direct increase of biological capacity. Another method for altering metabolism involves using tiny metabolite molecules to control the biological processes that produce oligosaccharides and glycoconjugates (Saeui et al., 2015). New metabolic routes that go against natural metabolism have also been introduced in metabolic engineering, highlighting the significance of integrating new metabolic pathways and using metabolic flux to affect cell physiology and achieve desired metabolic control (Baker et al., 2013).

Microorganisms, particularly bacteria have long been the primary source of several beneficial therapeutic products, such as antibiotics, antiviral medications, anticancer metabolites, enzymes and chemicals that inhibit enzyme activity, and therapeutic proteins (Abu-Qarn et al., 2008; Agatemor et al., 2019). Given that metabolism is a fundamental phenomenon that affects all living things and is necessary for a cell to regulate free energy obtained from organic and other sources. Therefore, recent research focussed on implementing metabolic engineering to improve strains of bacteria that produce useful metabolites, which can then be commercialized (Natarajan et al., 2020). This field of study is even more fascinating since live organism strains that become better through mutation and genetic engineering offer a huge chance to produce a novel product (Singh et al., 2019). Designer glycosylation pathways installed on metabolically altered bacteria are cutting-edge manufacturing platforms for the supply of many structurally changed medicinal medicines, dietary supplements, and vaccinations (Agatemor et al., 2019). Moreover, metabolic engineering facilitates relatively rapid and economically successful methods.

First, comprehend why using microbial cells rather than eukaryotic host cells is more advantageous for glycol engineering (Pandey and Gupta, 2022). It is important to emphasize that microbial cells do not naturally possess the metabolic glycosylation mechanism or rely on protein glycosylation. Specifically, both N-linked and O-linked glycosylation are essential for cell viability in eukaryotes (Reily et al., 2019). Besides, glycosylation plays a substantial role in the

growth and development of cellular structure and metabolism (Lehle et al., 2006). Fundamentally, it is always challenging to alter the natural metabolism of the glycosylation pathway through genetically engineered gene insertion and deletion, as well as gene overexpression for the production of the glycosyltransferase enzyme. This is because these modifications have a noteworthy harmful effect on the fitness of the host. Bacterial cells are able to overcome these issues because they lack native metabolic glycosylation mechanisms. In a cell, prokaryotic host cells make it easier to introduce orthogonal protein glycosylation pathways without endangering native glycosyltransferases, leading to more uniformly glycosylated protein by-products (Natarajan et al., 2018).

However, introducing a glycosyl pathway into a prokaryotic host is not a simple task. The absence of native glycosyl pathways presented significant engineering challenges. These include incorporating functional pathways for glycosyltransferases so they can catalyze preferred glycan biosynthesis on a protein target or a lipid carrier and gathering the required amount of essential building block molecules, such as acceptor proteins and nucleotide. Moreover, glycans synthesis in microbes is a carbon and energy incentive process (Ruffing and Chen, 2006). The various interconnected pathways that make up the crucial sugar nucleotide synthesis must be aligned for the systematic availability of building blocks, which poses some very difficult metabolic engineering problems. N-linked protein glycosylation pathway of *Campylobactor jejuni*'s was discovered, which significantly boosted bacterial glycoengineering (Szymanski et al., 1999). Later, scientists functionally inserted the full *C. jejuni* protein glycosylation locus (pgl), which codes for the entire N-glycosylation pathway, into a desirable *Escherichia coli* strain. Undecaprenyl pyrophosphate (Und-PP), a native lipid donor for *C. jejuni* glycans, is transferred to an acceptor protein aspargine residue in *E. coli* by the PglB oligosaccharyltransferase (OTase). Additionally, research has shown that PgIB is compatible with a variety of glycan substrates, suggesting that PgIB has relaxed specificity towards glycan substrates (Hug and Feldman, 2011). This enables the introduction of the desired glycan precursor moiety and the production of customized recombinant bacterial glycoproteins (Natarajan et al., 2018).

The idea behind metabolic engineering is to use non-natural tiny building blocks, or monosaccharides, to create larger biopolymers, including oligosaccharides and polysaccharides, through metabolic insertion. Such manipulation involves altering the enzymatic functions that participated in the biosynthesis of proteins and glycans using small molecules, as well as the

metabolic flux that drives the involvement of synthetic amino acids and monosaccharides in the glycan synthesis system of bacterial metabolism (Saeui et al., 2015). The methods for metabolic engineering-based bacterial glycoprotein engineering are described in this review, along with a new approach for producing customized glycans by overcoming the drawbacks and difficulties of microbial cell factories. Further, we focused on current advances in creating new artificial glycosylation pathways, identification of targets for metabolic engineering using genome analysis, construction of synthetic circuits that could be finely triggered for controlled expression of glycosylation pathway enzymes, and involvement of multidimensional genome editing with the latest engineering techniques used for the production of tailored glycans with anticipated physiochemical properties.

2. Metabolic glycoengineering

2.1. Metabolic engineering of glycoprotein biosynthesis

All life depends on modifying proteins, and almost 70% of eukaryotic species change proteins primarily by N-linked glycosylation (Abu-Qarn et al., 2008). Approximately 50% of all human proteins are thought to be glycosylated, meaning they have added sugar molecules. Sugar makes up to 90% of the mass of some glycoproteins. The synthesis of various therapeutic proteins requires glycosylation, a post-translational phenomenon that executes optimal folding of newly synthesized polypeptide into three-dimensional structures which impart stability and proper communication with the host immune system. Because eukaryotic cell lines are adept at executing sugars attaching to desired conformations, they are initially used for industrial scale glycotherapeutic manufacturing. For example, insulin has been derived from the pancreas of humans and other animal sources. But this could not be sufficient to meet the present rapid demand for therapeutic proteins. Along with on-going development in genetic engineering techniques, it has become a far more favorable platform to express a recombinant protein in different host systems such as bacteria, yeast, insects, plants, and mammalian cells (Jaffe et al., 2015). However, the recent finding of a functional N-linked glycosylation pathway in *C. jejuni* and its successful expression into the well-explored gram-negative bacterium *E. coli* facilitates a great opportunity to produce a variety of glycans and glycoproteins in an economically cheaper way in a most genetically flexible host (Piirainen et al., 2022). The trisaccharide Galβ-4GlcNAcβ-4GlcNAc terminal motif N-acetyllactosamine served as a key acceptor for α-1,3 fucosyltransferase (α-(1,3)-FucT). The

pathway of *E. coli* DHZ6 for the synthesis of the Lex-containing tetrasaccharide Galβ-4(Fucα-3)GlcNAcβ-4GlcNAc was successfully engineered (Figure 1A). The strain expresses the chitinoligosaccharide synthase (nodC gene), the truncated chitinase (chiA gene), and the α-(1,3)-FucT (futA gene). The chitinoligosaccharide synthase NodC generated chitinpentaose, which was subsequently breakdown into chitobiose and GlcNAc by Chitinase A1 (Dumon et al., 2006). Chitobiose then acts as an acceptor for β-(1,4)galactosyltransferase (β-(1,4)-GalT), which is encoded by lgtB, to synthesize Galβ-4GlcNAcβ-4GlcNAc. This compound is subsequently converted into 3 by α-(1,3)-FucT, which is encoded by futA. The targeted product was produced by glycosylation of chitobiose, which served as an acceptor for Galβ4T, encoded by lgtB, to form Galβ-4GlcNAcβ-4GlcNAc. Chitobiose is produced in the cytoplasm of bacteria by rhizobial chitin-synthase (nodC gene) and the chitinase A1 gene (chiA), both of which were introduced from *Bacillus circulans* into an engineered *E. coli* strain. Finally, Galβ-4GlcNAcβ-4GlcNAc is converted into α-(1,3)-FucT by *Helicobacter pylori's* α3 fucosyltransferase (futA gene)

Gal is typically transformed into galactose-1-phosphate (Gal-1-P) by the galactose kinase that is encoded by the galK gene once it has entered the cytoplasm. A galK-defective host strain (GLK) was created by causing the galK gene disruption in DC strain that prevent Gal catabolism and allow it to be utilised exclusively as acceptor. Galβ-4(Fucα3)GlcNAcβ-3Gal was produced from exogenously supplied Gal by a GLK in galactokinase activity and upregulating the expression of β3-N-acetylglucosaminyltransferase (iGnT; encodes by lgtA gene) (Figure 1B). Gal entered the cell through the Gal permease (galP gene). It was completely converted into Galβ-4GlcNAcβ-3Galβ by β-(1,3)N-acetylglucosamine transferase (β-(1,3)GlcNAcT; encodes by lgtA) and β-(1,4)GalT. Then, Galβ-4GlcNAcβ-3Galβ was mainly fucosylated by FutA into Galβ-4(Fucα-3) GlcNAcβ-3Gal (Dumon et al., 2006). E. *coli* was engineered with heterologous expression of *H. pylori*'s FucT and *Neisseria meningitidis*'s lgtAB genes for in vivo production of fucosylated-N-acetyllactosamine oligosaccharides (Figure 2). Downregulated genes in the biosynthetic pathway include wcaJ (involved in colanic acid synthesis) and lacZ (allowed lactose internalisation by the specific permease LacY). GlcNAcT and GalT, which are used to glycosylate lactose, were encoded by the gene lgtAB. Lacto-N-neotetraose (LNnT) is a possible acceptor for α-1,3 fucosyltransferase, resulting in lacto-N-neo-fucopentaose (LNnFP) and lacto-N-neo-di fuco-hexaose (LNnDFH). Thus, an *E. coli* strain that coexpresses the *N. meningitidis* genes lgtAB and fucT should be able to synthesize fucosylated derivatives of LNnT from lactose (Dumon et al., 2001). The catalytic

domain of the mouse glucuronyltransferase involved in HNK-1 carbohydrate biosynthesis. Yavuz and colleagues assessed ability of mouse β-glucuronyltransferase (GlcAT-P) to function in the *E. coli* cytoplasm (Yavuz et al., 2008). The product is synthesised by the mouse glucuronyltransferase catalytic domain by expressing it in an engineered strain of *E. coli*. Lactose is utilized as a carbon source and internalised by lacY (β-galactoside) and degradation is prevented by removing the lacZ gene. It is glycosylated by the β-(1,3)GlcNAcT enzyme from *H. pylori* and the β-(1,4)GalT gene from *N. meningitidis*. The β-(1,4)GalT gene from *N. meningitidis* and the β-(1,3)GlcNAcT enzyme from *H. pylori*, both glycosylate glucuronylneolactotetraose (GlcAnLc4). Alternately, glucuronyltransferase can use endogenous Lac and nLc6 as substrates, resulting in glucuronyllactose (GlcALac) and glucuronylneolactohexaose (GlcAnLc6), respectively. The synthesis of UDP-GlcA is catalysed by UDP glucose dehydrogenases from the *E. coli* strains, namely K5 and K-12 (Figure 3).

Antonie and colleagues reported that high-density culture of *E. coli* strains overexpressing the corresponding glycosyltransferase and sugar nucleotide synthase genes (Figure 4) can efficiently produce the carbohydrate moiety of gangliosides GM2 (GalNAcβ-4(NeuAc-3)Galβ-4Glc) and GM1 (Galβ-3GalNAcβ-4(NeuAc-3)Galβ-4Glc) and their respective asialo derivatives, namely aGM2 (GalNAcβ-4Galβ-4Glc) and aGM1 (Galβ-3GalNAcβ-4Galβ-4Glc) (Antoine et al., 2005). Two metabolically engineered strains of *E. coli* have been developed to produce the carbohydrate moieties of gangliosides, namely GM2 (GalNAcβ-4(NeuAcα-3)Galβ-4Glc and GM1 (Galβ-3GalNAcβ-4(NeuAcα-3)Galβ-4Glc (Antoine et al., 2003). Lactose and N-acetyl-D-neuraminic acid (NeuAc), which are transported inside the cell by the permeases LacY and NanT, protect it from further degradation caused by the inactivation of the β-galactosidase (LacZ) and aldolase (NanA) genes. Cytidine 5′-monophospho-N-acetylneuraminic acid (CMP-NeuAc) synthase transforms NeuAc into CMP-NeuAc, a nucleotide-activated form, which is subsequently transferred onto lactose by α-2,3-sialyltransferase (Lst) gene, resulting in sialyllactose. *N*-acetylgalactosamine (GalNAc) transferase (CgtA) uses the endogenous pool of UDP-GalNAc created by the recombinant UDP-GlcNAc C4 epimerase (WbpP) for providing UDP-Gal for galactosyltransferase to catalyse the glycosylation of sialyllactose to produce II^3Neu5AcGgOse$_3$. This substance reacts to produce II^3Neu5AcGgOse$_4$ and acts as an acceptor for β-(1,3)-GalT.

The gene of glycosyltransferase that utilizes lactose as acceptor, expressed *E. coli* result in glycosylation of intracellular lactose. CMP-Neu5Ac synthase expressing *E. coli* JM107-nanA and

the α-2,3 sialyltransferase genes from *N. meningitides* utilized to synthesize 3′-sialyllactose, Neu5NAcα2-3Galβ1-4Glc (Figure 5A). Both CMP-Neu5Ac synthase and α-2,3 sialyltransferase are produced, which enable the activation of NeuAc into CMP-Neu5Ac intracellularly and its subsequent transfer onto lactose. Meanwhile, high density cultivation of β-galactosidase-negative strains that overexpressed the lgtA gene of *N. meningitides* produced trisaccharide GlcNAcβ1-3Galβ1-4Glc by 6 g L^{-1} and prevents lactose degradation due to the cell's lack of β-galactosidase activity (Figure 5B). Further, when lgtB gene *N. meningitides* coexpressed with lgtA, the target trisaccharide converted to lacto-N-neotetraose and lacto-N-neoheaxose with the yield of 5 g L^{-1} (Priem et al., 2002).

Metabolic pathway engineering in *E. coli* for the production of chitobiose is depicted in Figure 6. Co-expression of the chitinase gene chiA and the rhizobial chitinoligosaccharide synthase gene nodC resulted in the synthesis of N^{I},N^{II}-diacetylchitobiose (chitobiose) in *E. coli* (Cottaz and Samain, 2005). Chitobiose was mainly recovered by being phosphorylated by the PTS permease ChbBCA. Intracellular chitinbiose was transformed into the trisaccharide Galβ4GlcNAcb4GlcNAc when the gene lgtB for β-1,4-GalT was expressed. This compound could operate as an acceptor for glycosyltransferases that recognize the terminal N-acetyllactosamine structure.

In prokaryotes, the UDP-GlcNAc biosynthetic route has been thoroughly characterised. Three enzymes work together in four successive enzymatic processes to convert fructose-6-phosphate (F-6-P) into UDP-GlcNAc (Figure 7). First, glucosamine-6-phosphate synthase (GlmS) converts F-6-P to glucosamine-6-phosphate (GlcN-6-P). The N-terminal domain of GlmS catalyzes the hydrolysis of L-glutamine to L-glutamate. Ammonia was used in this process, while the C-terminal domain was in charge of producing GlcN-6-P by using the ammonia. Second, GlcN-1-P was produced from glucosamine-6-phosphate. In a ping-pong bi-mechanism that uses glucosamine-1,6-bisphosphate as an intermediary, phosphoglucosamine mutase (GlmM) in a phosphorylated state catalyses this step. Acetylation of GlcN-1-P to form GlcNAc-1-P and conversion of GlcNAc-1-P (plus UTP) to UDP-GlcNAc (plus pyrophosphate) was catalysed by the bifunctional glucosamine-1-phosphate acetyltransferase/N-acetyl glucosamine-1-phosphate uridyltransferase GlmU. The acetylation reaction was catalysed by the C-terminal GlmU domain, whereas the uridylation reaction is catalysed by the N-terminal domain (Gauttam et al., 2021).

Metabolic pathway engineering in *E. coli* for the production of globotriose and globotetraose is presented in Figure 8. The lgtC gene from *N. meningitidis* was used to transfer α-1,4Gal transferase to *E. coli*. Two additional genes, WbpP from *Pseudomonas aeruginosa* (encoded by UDPGalNAc C4 epimerase) and IgtD from *Hemophilus influenzae* RD (encoded by β1,3GalNAc transferase), were overexpressed in *E. coli* to synthesize globotetraose. Further globotriose degradation is prevented by the absence of the melA gene, which encodes α-galactosidase. The LgtD converts globotriose to globotetraose, which encodes β3GalNAc transferase (Antoine et al., 2005).

Molecular engineering studies suggest that *E. coli* successfully glycosylates many proteins, including those of bacterial and eukaryotic origin, by modifying sugar structures. The fundamental eukaryotic sugar trimannosyl-chitobiose has been generated by integrating diverse genes into cells (Strutton et al., 2019). A set of orthogonal pathways expressed in *E. coli* that employed O-linked protein glycosylation (Figure 9), in which cancer-associated mucin-type glycans, such as Tn, T, sialyl-Tn, and sialy-T are attached at serine residues of human O-glycoproteins derived acceptor motifs (Natarajan et al., 2020). The Und-PP-linked GalNAc formed by *Acinetobacter baumannii* 17978 (*Ab*PglC) that utilizes activated nucleotide sugar base UDP-GalNAc, which was provided by *C. jejuni* UDP-Glc(NAc) 4-epimerase (*Cj*Gne). Und-PP-GalNAc was extended by enterohemorrhagic *E. coli* O104 (*Ec*WbwC) by a single Gal residue, resulting in lipid-linked Gal-1,3-GalNAc. The native *E. coli* flippase Wzx flips the lipid-linked oligosaccharide to the periplasmic face of the cytoplasmic membrane. Then an O-oligosaccharyltransferase (*O*-OST) like PglO from *Neisseria gonorrhoeae* (*Ng*PglO) or PglL from *N. meningitidis* (*Nm*PglL) transferred the preassembled T antigen glycan all at once to a serine amino acid on a Sec pathway-exported acceptor protein. It has emphasized that the production of Tn-modified acceptor proteins can be achievable in the absence of *Ec*WbwC, and that Galβ1,3GalNAc can also be further elaborated with other sugars like NeuNAc before being transferred to proteins. Further, using the same bacteria crude cell extract was used to express an antigenically authentic Tn-human mucin 1 (Tn-MUC1) glycan which demonstrated reactivity with antibody 5ES, which has been used particularly for cancer-associated glycoforms of MUC1.

Since glycans are secondary gene products that are generated via various metabolic steps regulated by different enzymes. The process is an energy incentive and exerts a greater metabolic burden on the bacterial host cell. So the more convenient approach is using metabolic engineering

and the inverse metabolic engineering method to focus target pathways and make necessary gene manipulations in them for improved productivity. It was reported that this approach successfully enhanced the desired protein amount with the structure of the preferred glycan without much loss in the total yield of recombinant protein. For example, overexpression of IcL enzymes in *E. coli* increases glycosylation efficiency 3-fold. In the glyoxylate pathway, IcL chops isocitrate into succinate and glyoxylate. Also, it was documented that the process via the glyoxylate pathway conserves much carbon utilization compared to the full citric acid cycle pathway, thus, this conserved carbon could be used for glycan precursor synthesis. Similarly, researchers have employed inverse metabolism strategies to identify *E. coli* cells showing an improved ability to N-glycosylate (Pandhal et al., 2013). They used DNA fragments from *E. coli* W3110 and expressed them into *E. coli* CLM24 cells which performed N-glycosylation to create a plasmid-based genomic library. Potential cells were screened using GalNAc-specific lectin peroxidase, which highlighted the improved strain. These strains were sequenced and selected for an extra-chromosomal insert. The protein was then labeled with heavy isotopes to identify abundant glycosylated proteins. They observed a 7-fold increase in AcrA production when they tested the effect of ptsA gene overexpression in *E. coli* cells. The process is triggered when the phosphoenol-pyruvate phosphorylated PtsA gene later transfers this phosphoryl group to a histidine phosphor carrier protein, which initiates the cascade process. This important pathway in *E. coli* participates in the phosphorylation of N-acetylglucosamine and a variety of sugars, which is required in bacterial glycosylation (Pandhal et al., 2013). Protein glycosylation depends on lipid carriers and nucleotide–activated sugars as precursor molecules in engineered *E. coli*. Therefore, plausible strategies for improving glycosylation efficiency are considered such as increased glycosylation efficiency by supplementing GlcNAc in bacterial culture media, where genes associated with glycan precursor synthesis and lipid carrier were recognized as rate-limiting factors (Kampf et al., 2015).

The great capacity of *C. jejuni* PgIB to transfer a variety of Und-PP-linked glycans residues has been reported (Valderrama-Rincon et al., 2012). This is the first time that eukaryotic N-linked protein glycosylation machinery has been assembled in *E. coli*. A series of glycosyltransferases (WecA, Alg13, Alg14, Alg1, and Alg2) involved in sequential glycan assembly on the lipid carrier Und-PP in the periplasmic face of the inner membrane. WecA (undecaprenyl-phosphate α-N-acetylglucosaminyl 1-phosphatetransferase) an endogenous glycosyltransferase (GTase) that

transfers GlcNAc1 phosphate to undecaprenyl phosphate. Specifically, an enzyme β1,4GlcNAc transferase of *S. cerevisiae*, which is made up of the Alg13 and Alg14 subunits, was utilized to add the second GlcNAc residue to GlcNAc-PP-Und. The β-1,4-mannosyltransferase (Alg1) from *S. cerevisiae* adds the first mannose to the glycan, and the bifunctional mannosyltransferase (Alg2) carries out the addition of both a α-1,3-mannose and a α-1,6-mannose in a branched configuration. Flippase used to transfer assembly of lipid and sugar residues towards the periplasmic phase, where they were transferred to the asparagine residue of acceptor proteins by a single subunit integral membrane protein, which shows *N*-OST activity to transfer sugar moiety to protein residue both co-translationally or post-translationally.

The modified *E. coli* strain showed glycosylation of particular asparagine residues in desired proteins including the Fc domain of an IgG, a scFv, and human growth hormone (hGH) variant. A flow cytometry analysis was applied to engineer *E. coli* for enhanced eukaryotic protein glycosylation (Glasscock et al., 2018). Initially, they identified the previously developed synthetic pathway in *E. coli* that facilitates site-specific glycosylation of eukaryotic proteins with mannose3-N-acetylglucosamine2 (Man3GlcNAc2), which presented as the essential assembly in all human N-linked glycans. The modification in the pathway was engineered by introducing multiple GTases from yeast, oligosaccharyltransferase, and PgIB from *C. jejuni*. This glycosylation pathway follows three consecutive steps, including glycan biosynthesis, glycan membrane translocation, and transfer of glycans onto polypeptide acceptor sequons. But engineered *E. coli* cells showed relatively low levels of glycosylated proteins, less than 1% (50 μg L^{-1}). The authors also identified that low glycosylation is due to the low production of lipid-linked Man3GlcNAc2. They identified and specifically improved the expression of certain genes using flow cytometry analysis, such as overexpression of two different endogenous biosynthetic enzymes that participate in GDP-mannose precursor and decreased the expression level of heterologous yeast GTases. This manipulation imparts a 4-fold enhancement in the level of Man3GlcNAc2, ultimately leading to a 50-fold rise in the intracellular level of Und-PP-linked Man3GlcNAc2.

A recent study has explored different process engineering and genetic engineering approaches to enhance recombinant N-linked glycosylation in engineered *E. coli* with *Campylobacter*-derived PglB (Pratama et al., 2021). By restricting oxygen supply or using an E. coli strain with limited disulphide-bond isomerase and oxidoreductase activity, the authors altered the target protein's cleavage location and secretion signal and altered how the protein folded in the

periplasm. Through these modulations, the research team was able to increase the membrane residence time of the desired protein for glycosylation and achieve a 2-fold increment in glycosylation efficiency. In the subsequent experiment, they reported that supplementing the production media of the oxidoreductase knockout strain with the chemical oxidant cystine demonstrated improvements in total protein production and cell fitness, leading to an enhanced titer of glycoprotein and a high level of glycosylation efficiency. This study suggested that by mimicking the native hosts (*C. jejuni* and mammalian cells), the cellular process of coordination among protein folding, its glycosylation and translocation in the heterologous host could potentially improve the production of heterologous recombinant N-glycoprotein.

Bacterial glycoengineering and cell-based production of modified therapeutic glycoproteins homogenously have remained a great challenge due to maintaining cell viability, sufficient production of glycosylation components, and a new set of engineered enzyme expressions. These issues and the development of an integrated cell-free glycoprotein synthesis method, which offers several potential advantages, were addressed (Jaroentomeechai et al., 2018). This open system allows direct manipulation of the biochemical system of interest. The new component can easily be added for target component synthesis, and the process bypasses cell viability constraints, the need for OSTs, and extraction of LLO using organic solvent from bacterial membranes. The CFGpS system developed was supported by (i) cell-free protein expression and N-linked glycosylation prepared from the extract (crude) of the glycol-optimized *E. coli* strain CLM24 and (ii) glycosylation components in OSTs and an LLO-enriched extract from CLM24 facilitate co-activated protein synthesis and N-linked glycosylation in the reaction system. The resulting mixture makes possible one-pot site-specific glycosylation of the target protein.

The natural glycoprotein glycosylation requires enzymatic transglycosylation at GlcNAc-Asn residues. Therefore, researchers have explored several endo-β-*N*-acetylglucosaminidases (ENGases) transglycosylation activity to synthetically modify glycoproteins. ENGases in addition to their hydrolytic activity possess ability to glycosylate GlcNAc-containing proteins. Using site directed mutagenesis, followed by activity screening result in discovery of ENGase-based glycosynthases that can able to incorporate highly active synthetic oxazolines sugar for transglycosylation. This system of oxazoline donors and ENGases, in which product hydrolyzed catalytic amino acids were removed via mutation technique, efficiently attached homogeneous complex-type oligosaccharides to IgG with adequate yield (Wang, 2011). Through this approach,

artificial oligosaccharides harboring structural biology probes like stable isotopes or selenium can, at a definite position, conjugate with proteins (Búa et al., 2015).

2.2. Metabolic engineering of oligosaccharides biosynthesis

As glycobiology developed, it became clear how oligosaccharides were put together inside of cells with proteins and lipids through glycosylation pathways and participated as important surface components including signalling molecules, receptors, and surface indicators. Many of these surface markers were found to be participated in biological communications in a wide range of health and disease signals, from cancer to influenza. As a small change in oligosaccharide displays significant immune system modulation, these "oligosaccharide codes" help to regulate and modify the important function in diseases. Therefore, glycans are also considered companions to proteins and nucleic acids because all of them are key players in transmitting and storing biological information in virtually all organisms. However, the study of glycans and the synthesis of tailored glycans is still challenging because the glycan synthesis process is not template dependent as in the case of proteins biosynthesis and nucleic acids. Glycans occur via a complicated and dynamic process of chain elongation or trimming of glycans through different glycosyltransferase and glycosidase enzymes (Sun, 2018). Now, metabolic oligosaccharide engineering (MOE) has arisen as a promising method for manipulating glycosylation. In the 1990s, it was detected that enzymes that take part in glycosylation salvage pathways occasionally catalyze unnatural alterations in the monosaccharide substrate structure (Wang et al., 2022). This enlightens the idea of assembling desired modified monosaccharides into glycans for tailored glycan production.

In the MOE method, tailored glycans are produced by introducing non-natural monosaccharide analogs into biosynthetic salvage pathways. The cells transform these non-natural sugars into activated nucleotide phosphate donors, which are later assembled into targeted glycan structures by glycosyltransferase enzymes. Currently, a diversified range of non-natural sugar analogs such as GalNac, GlcNAc, sialic acid, NeuAc, and fucose have been successfully integrated into glycoconjugates that appear on the cell surfaces (Sminia et al., 2016). Introduced analogs get inserted into all glycans that utilize monosaccharides of interest for chain building; this allows parallel track down of multiple glycan structures. However, this will precisely render the detection of particular structures that are different from neighboring glycans based on motifs and glycosidic bonds. Since both natural and non-natural sugar substrates compete for incorporation into cellular

glycan synthesis, the overall detection sensitivity of tailored glycans was reduced. Therefore, the desired foreign analog must contain a small non-natural moiety so that glycosylation pathway enzymes can efficiently recognize them and incorporate them into glycans (Kufleitner et al., 2023). Now a day's variety of chemical probes including inert alkyl chains, and reactive chemical groups such as thiols, azides, alkynes, ketones, isonitriles and cyclopropenes used to install in non-natural analogs (Sminia et al., 2016). The reactive groups facilitate the covalent attachment of different reporters' molecules such as fluorescent dyes, biotin, cross-linking agents, and radio-labeled isotopes. The major advantage of chemical probe-based structural analogs in MOE is that the chemicals employed as probes allow bioconjugate reactions and easy profiling of labeled glycans. Such chemical tags offer a variety of purposes, ranging from improved pharmacological and biological properties to drug delivery through new modes of glycans (Campbell et al., 2007).

The breakthrough in MOE begins with the discovery of azide and alkyne conjugates for metabolic labeling. An amide bond is created by the coupling of an azide and a specially designed triarylphosphine in a ligation that is modelled after the Staudinger reaction (Saxon and Bertozzi, 2000). In the most popular copper-catalyzed azide-alkyne cycloadditions (CuAAC) reaction, substrates azide and alkyne respond quickly to produce stable triazoles. To remove excess copper from *in vivo* systems, two protocols were developed, one is an amine-triazole-based ligand that limits the copper requirement by accelerating the CuAAC reaction, and the other method is strain-promoted azide-alkyne cycloadditions (SPAAC) reaction, used to escalate the reaction kinetics in a copper-free fashion (Agard et al., 2005). Later, several photo-controlled versions of the Staudinger-Bertozzi ligation were discovered and used to control bioconjugation reactions within the living system.

2.3. Chemical probes and their tagging mechanism in bioconjugation reaction

As previously discussed, a variety of chemical handles are used to tag labeled glycans after MOE experiments, such as aliphatic analogues, natural glycans, ketones, thiols, alkynes, azides, terminal alkenes, sialic acids, xylose, trehalose, diazoketones, cyclopropenes, diazirines, and norbornenes (Figure 10-16; Table 1). The basic techniques behind synthesizing these chemical handles include amide coupling or other complex synthetic strategies. Because acetyl esters aid in the probe's passive absorption, each manufacturing technique focuses primarily on the final product when using an acetylated monosaccharide based chemical probe supplied to the MOE target cell. Acetylated monosaccharide probes are internalized by the cell, and after their internalization, the

acetyl moieties are dissolved by cytosolic esterases. Then the probe enters the glycan salvage route, where it is digested and converted into a nucleotide-activated donor substrate. Glycosyltransferase enzymes use this substrate in the cell's ER and Golgi apparatus for glycosylation processes. In this manner, a range of intricate cellular glycan structures, including O-glycans, N-glycans, and glycolipids, incorporate the labeled donor sugar probe. Later, such modified chemical handle probes were used for targeted monosaccharide profiling in the complex glycans of cells (Sminia et al., 2016).

2.4. Metabolic oligosaccharide engineering in bacteria

Glycans are essential carbon source for bacterial growth and viability. Bacteria synthesize a wide range of unique glycans, which presents them as the best host for developing tailored oligosaccharides and novel antibiotics. Therefore, the metabolic labeling of bacterial glycans is gaining much attention in this research field. MOE can employ diverse bacteria strains, including pathogenic strains, such as *Haemophilus ducreyi*, *H. pylori*, and *C. jejuni* and non-pathogenic strains like *E. coli* and *Bacteroides gragilis*. On the other hand, synthetic sugar analogs degraded during the catabolic process in bacteria, resulting in low competing probe incorporation in glycans. Using a high concentration of synthetic probes (azido-glycans) to overcome the above-illustrated problem, bacteria also demonstrate low toxicity and high tolerance against chemical probes. Bacteria better tolerate even the CuAAC reaction that generates copper compared to other host cells. Therefore, both CuAAC and SPAAC reactions have been successfully used for MOE in bacteria. Many non-natural glycans entail immense and complex routes to systematically produce the probe, which requires labeling targeted microbial glycans. For example, pseudaminic acid structurally related to sialic acid and found as an O-linked post translational modified flagellin protein in *H. pylori* and *C. jejuni*. Aazide-labeled pseudaminic acid (Az-Pse) into the bacterial glycan of the sialic acid class was successfully incorporated *C. jejuni* mutant cells lacking the Pse pathway showed the presence of Az-Pse on the flagella when fed with the azido-Pse precursor, 6-deoxy-AltdiNAz which condensed with phosphoenolpyruvate by pseudaminic acid synthase to give Pse (Liu et al., 2009).

Using metabolic engineering, a research group triggered an artificial immune response against *H. pylori*, which causes human stomach ulcers (Kaewsapsak et al., 2013). They first incorporated azide containing sugar precursor, peracetylated *N*-azidoacetylglucosamine

(Ac$_4$GlcNAz) selectively onto *H. pylori* cells. Then azide group ligated with phosphines conjugated with immunostimulant, 2,4-dinitrophenyl (DNP) via Staudinger ligation resultin display of antigenic azido-functionalized glycoproteins on H. pylori cells. In contrast, mammalian cells did not incorporated chemical labeled Ac$_4$GlcNAz in cell surface glycans. Subsequently, bacteria were supplemented with anti-DNP antibodies, and the effect was analyzed by using an antibody-dependent cell-mediated cytotoxicity assay. The result indicated 2-fold bacterial cell deaths, suggesting metabolically engineered glycans could generate an artificial immune response against human pathogenic bacteria.

A recent study has attempted to improve the feeble alkyne-tagged MOE reagents Ac4GalNAlk 1 and Ac4GlcNAlk 2 into a competent chemical tag to probe cell surface protein glycosylation (Cioce et al., 2021). They reported that surpassing bottleneck metabolic steps by using mut-AGX1 potentially enhanced the biosynthesis of nucleotide sugars UDP-GalNAlk and UDP-GlcNAlk and boosted bioorthogonal cell surface labeling by 2-fold, which was substantially more extensive labeling compared to known azide-tagged MOE reagents. This experiment further expanded the toolbox of chemical glycobiology.

Another strategy to express tailored glycans for the production of essential therapeutic agents is engineering bacterial cells for peptidoglycan (PG) synthesis. PG of bacteria is a necessary biopolymer for their survival and a bacterial cell wall component. It also maintains cell shape, aids in cell division, small molecule recognition, and signaling, and aids in bearing osmotic pressure inside the cell. The sugar units GlcNAc and MurNAc that make up the bacterial PG layer are linked by brief peptide chains that are bonded to the lactoyl acid residue of MurNAc (de Pedro and Cava, 2015). These molecular blocks combine to form a variety of PG, which directly impact human health due to their immunogenic properties. PG biosynthesis is a complex and conserved cellular process that engaged distinct enzymatic steps. The first step in the synthesis of bacterial PG occurs in the cytosol, where nucleoside-activated sugar units UDP-MurNAc-pentapeptide and UDP-GlcNAc are produced. On the inner layer of the plasma membrane, these UDP-linked sugar units are used to create the precursors for PG synthesis, undecaprenyl-pyrophosphorylase-MurNAc (pentapeptide)-GlcNAc lipid II is then transported through the cell membrane and involved in the polymerization process that produces PG. In the final phase of PG synthesis, GTase catalyzes the elongation of the PG glycan chain. This is followed by the Tpase reaction, which creates crosslinks between the synthesized PG glycan chains and other peptides.

The pathways involved in PG polymer biosynthesis for bacterial cell wall formation are an ideal target for developing efficient antibiotics because these pathways are essential for bacteria and are absent in mammalian cells. Numerous in vivo PG-labeling agents have been developed recently to investigate PG production in order to clarify the antibiotic's mechanisms of action and examine host-pathogen interactions (Hira et al., 2020). The creation of fluorescent amino acids, namely clickable D-amino acids, and dipeptides of D-amino acids that can be combined with bioorthogonal and provide tools to observe PG assembly. MurNAc/GlcNAc kinase (AmgK) and MurU, two PG recycling enzymes, were utilized in a chemoenzymatic synthesis method to change MurU (Taylor et al., 2020). These enzymes utilize and process the bioorthogonal handles containing MurNAc mimics. In another study, different variants of UDP-MurNAc were used to fluorescently label the whole bacterial cells. However, only a few studies have been done on GlcNAc fluorescent-based PG labeling, however, this strategy is still poorly explored. Researchers successfully fluorescently labeled PG by employing the ketone moiety and incorporated them into the N-acetyl group of GlcNAc (Sadamoto et al., 2008). Recently, a study used the bioorthogonal handle GlcNAc to label PG. Using the glycosyltransferase OleD, their team created a one-step simple synthesis method for UDP-GlcNAc (Xu et al., 2022) (Figure 17). Since some of the reactions that the glycotransferases catalyze are reversible, they are thermodynamically unfavorable for the synthesis of NDP-sugars. To solve this issue, they use metabolic engineering. They added the metabolite GlcNAz-CNP and a variant GTaseOleD TDP-16, which catalyzed NDP-sugar formation from a 2-chloro-4-nitrophenyl glycoside donor and simultaneously in-vitro integrated GlcNAz. Further investigation revealed fluorescently labeled lipid II that had been incorporated into PG via the bifunctional class A penicillin-binding proteins' DD-transpeptidase activity. Their method explored the heterologous expression of OleD as an efficient strategy for PG synthesis by involving GlcNAz metabolism in vivo.

2.5. Metabolic engineering of nucleotide sugar biosynthesis

Surprisingly, a wide range of glycans and their conjugates may be found in nature. Nonetheless, in the live system, glycosylation pathways use just nine precursor saccharides as a building block for glycoprotein and glycolipid production. GlcNAc, GalNAc, galactose, glucose, glucuronic acid, mannose, xylose, fucose, and N-acetylneuraminic acid are some of the most prevalent precursors. However, the availability of nucleotide-activated sugars as a high-energy substrate for

glycosyltransferase activities is required for glycosylation and glycan synthesis. To integrate into complex glycan chains, sugar precursors must be activated before the building blocks into nucleotide donors. Sugar nucleotides are classified into two categories based on the nucleoside attached, such as NMP and NDP. For example, cytidine monophospho-N-acetylneuraminic acid is an NMP sugar, while uridine or adenosine-diphosphate glucose, guanosine-diphosphate mannose, and UDP-GlcNAc are NDP sugars. The most prevalent nucleotide-active sugar donors are UDP (uridine 5'-diphospho-α-D) and UDP-linked amino sugars, which play a major role in the formation of bacterial cell walls as well as glycolipids, glycoproteins, oligosaccharides, and glycosides (Wang et al., 2022). In addition, both eukaryotes and prokaryotes depend on UDP-GlcNAc for several metabolic functions.

In much better approach, one-pot syntheses of nucleotide sugar can be achieved by using enzyme cascades to manufacture from readily available and inexpensive starting materials. A glycosylation process can be paired with a nucleotide sugar-producing cascade, and the nucleotide released is frequently recycled to replenish the nucleotide sugar. OPME (one pot, several enzymes) reactions are processes that involve numerous phases and several enzymes, and single OPME reactions can be used to generate more complicated systems. In initial step in enzymatic catalysis (kinase catalyzed) sugar get phosphorylation with a nucleoside triphosphate. Whereas in one pot synthesis kinase first produces a sugar-1-phosphate and a pyrophosphate forming enzyme finalize the synthesis. For example, to create the pyrophosphate bridge, UDP-GlcNAc has been synthesised via a kinase-dependent process using a pyrophosphorylase (Fischöder et al., 2019) or an uridylyl 1-phosphate transferase (Eisele et al., 2018). Initially, N-acetylhexosamine kinase (NahK), a wild type gluco-type 1-kinase was identified that can catalyse the production of GlcNAc-1-P from a GlcNAc acceptor and a 5'-ATP phosphate donor (Nishimoto and Kitaoka, 2007). Then Kinase-dependent routes for the synthesis of different nucleotide sugars, including UDP-Gal and UDP-GalNAc, GDP-mannose, GDP-fucose, and nucleotide sugars containing unnatural sugar moieties, have been identified (Fischoder et al., 2019; Tasnima et al., 2019). GDP-mannose has synthesized through a kinase-free pathway from mannose 1-phosphate (Pfeiffer et al., 2016). NahK has used to effectively convert unnatural GlcNAc/GalNAc analogues to 1-phosphorylated products, independent of the stereo-configuration at the C-4 location of the sugar ring (Zhao et al., 2010). The relaxed conformation for N-acyl chain modifications can allow the addition of bio-orthogonal functionalities loaded with additional tags and labels. In one pot synthesis of UDP-sugar

derivatives, NahK has combined with other two enzymes includes PmGlmU and PmPpA (Chen et al., 2013).

Sucrose synthase catalyses the conversion of sucrose to the suitable nucleotide-activated derivative of glucose (NDP-glucose) in the presence of a pyrimidine or purine nucleoside diphosphate (NDP). Kulmer and co-workers used polyphosphate kinases (PPK, a biocatalytic cascade from *Meiothermus ruber*) from class II and III of family 2 which catalyzes the conversion of UMP into UDP, and sucrose synthase from *Acidithiobacillus caldus* for established NDP-glucose production (Kulmer et al., 2017). UMP is much more cost-effective substrate than UDP. In a chemoenzymatic approach used for the synthesis of unnatural UDP-GlcNAc/UDP-GalNAc analogs based on method including chemical and enzymatic synthesis with GlmU (Guan et al., 2009). They successfully prepared facile library of unnatural sugar nucleotides with modification of diversified bio-orthogonal reactive groups, especially which involved functionalization of C-2 nitrogen group. It was reported that C4 position of GlcNAc/GalNAc is comparatively less reactive therefore efforts have been made to modify C4 positions to form unnatural sugars. For example, 4-OH group did not play significant role in GlmU recognition. Using 4F-GlcNAc-1-phosphate and 4F-GalNAc-1-phosphate analogues as GlmU substrates the production yield have been enhanced greatly compared to studies using UDP-GlcNAc pyrophosphorylase produced in *E. coli* JM109. Further this approach allowing considerably simplified purification processes (Feng et al., 2004).

Recently, researchers have demonstrated a significant accumulation of UDP-linked activated amino sugar, called UDP-GlcNAc both intracellularly and extracellularly by transforming the biosynthetic pathway of *C. glutamicum* via MOE strategies (Gauttam et al., 2021). In *C. glutamicum* GRS43 plasmids, they engineered *C. glutamicum* and overexpressed native *E. coli* genes such as *glmS*, *glmU*, and *glmM* in various combinations. These genes are in charge of producing the enzymes that catalyze the production of UDP-GlcNAc from the F-6-P substrate (Figure 7). However, the glucosamine-6-phosphate deaminase gene (nagB), which is required for glucosamine breakdown, is absent from strain GRS43. Subsequently, incorporation and increased expression of *glmS*, *glmU*, and *glmM* genes engineered in *C. glutamicum* efficiently encoded glucosamine-6-phosphate synthase, bifunctional glucosamine-1-phosphate acetyltransferase/N-acetylglucosamine-1-phosphate uridyltransferase, and phosphoglucosaminemutase, respectively. This increased UDP-GlcNAc concentration intracellularly was more than 50-fold, which is close to 20-fold greater than that of recombinant *L. casei* BL23. In contrast, the culture supernatant of

recombinant *C. glutamicum* strains produced 60 mg L^{-1} UDP-GlcNAc. This high concentration of UDP-GlcNAc facilitates downstream processing and provides product stability. Other research group used starch as starting material in a whole cell biosynthetic mechanism, where glucan phosphorylase generates glucose-1-phosphate, which gets converted into UDP-glucose by pyrophosphorylase. Further, NADH-dependent hydrogenase oxidized UDP-glucose into UDP-GlcA via hydroxymethyl group. An oxidase may recycle the NADH coenzyme. Then, another pathway to UDP-GlcA starts with glucoronic acid, bypassing the NADP-dependent oxidation step (Meng et al., 2019).

2.6. Metabolic engineering of polysaccharides biosynthesis

Functional polysaccharide polymers are well known for their high biological activity, diverse chemistries, and vast therapeutic applications (Prateeksha et al., 2022; Singh et al., 2018). Therefore, functional polysaccharides possess immense industrial importance as well. Some of the well-known functional polysaccharides are xanthan, heparin, alginate, chondroitin sulfate, etc. EPS is secreted extracellular by microorganisms into their surrounding growth medium (Prateeksha et al., 2019; Prateeksha et al., 2021; Singh et al., 2009; Singh et al., 2015). They form the majority of natural bacterial biofilms and host-pathogen interactions and serve as a protective barrier against unfavorable host environments such as pH, degrading enzymes, and immunological host defense molecules (Prateeksha et al., 2017; Singh et al., 2015a; Singh et al., 2012; Singh et al., 2015b). EPS biopolymers are widely used in food, cosmetics, and feed, as well as in medical and technical applications. Glucosaminoglycans (GAGs) are another class of higher eukaryotic polysaccharides that include four major classes, namely chondroitin sulphate, heparin/heparin sulphate, dermatan sulphate, and hyaluronan, which are primarily made up of HAs and hexosamines (Schwartz and Domowicz, 2023). The proteoglycans, and particularly their GAGs moieties showed interaction with hormones, growth factors, chemokines, morphogens, and other survival molecules impart crucial physiological effects, establishing their high biopharmaceutical applications. The current demand for functional polysaccharides necessitates the discovery of more diverse polysaccharides, which has sparked a lot of interest in biotech. Metabolic engineering facilitates the modification of distinct biosynthetic pathways for polysaccharide production in microbial cell factories. Applied metabolic engineering strategies are primarily based on (i) an adequate supply of nucleotide-linked sugars and donor availability; (ii) recombinant expression in

suitable bacterial host cells; (iii) optimization of substrate consumption by the recombinant host, and (iv) attenuation of competing for central carbon fluxes.

Homopolysaccharides like levan or dextran are commonly used in metabolic engineering, which typically relies on single enzymes that catalyze the polymerization of di- or oligosaccharide substrates extracellularly. *Bacillus amyloliquefaciens* was engineered by replacing the levan sucrose SacB natural promoter genes with the more promising artificial pgrac promoter (Gu et al., 2017). Moreover, secretion efficiency was enhanced by swapping the YncM peptide from *B. subtilis* with the signal peptide of *B. amyloliquefaciens*. The recombinant strain significantly improved levan titers concentration in fed-batch fermentations, reaching 102 g L^{-1}. In another work, lipopolysaccharide production in *Xanthomonas campestris* was inhibited as part of metabolic engineering techniques to increase the EPS titer (Schilling et al., 2020; Steffens et al., 2016). The resulting modification increased nucleotide sugars precursors and improved EPS yield by 26%. Impurities of xanthomonadin raise the cost of prodsugarxanthan gum from *X. campestris* CGMCC15155. Large volumes of ethanol are needed in the industrial manufacture of xanthan gum using *Xanthomonas campestris* CGMCC15155 because xanthan gum must be extracted from the fermentation broth and xanthomonadin impurities must be removed. A xanthomonadin-deficient strain of CGMCC15155 was created by introducing the *Vitreoscilla globin* (*vgb*) gene, under the control of the LacZ promoter, into the region of the pigA gene, which is responsible for xanthomonadin synthesis (Dai et al., 2019). Although the lack of xanthomonadins reduced xanthan gum yield, the *vgb* gene expression product increased *X. campestris* metabolism and xanthan gum production in a modified strain comparable to the wild-type strain. This reduced ethanol consumption significantly by 133.3% and overall cost of producing xanthan gum.

HA is the easiest GAG to produce recombinantly, as it does not follow post-polymerization modifications (Woo et al., 2019). The *C. glutamicum* strain was metabolically engineered at the genome scale to enhance HA titers (Du et al., 2021). They artificially expressed HA synthesis pathway genes in a *C. glutamicum* strain, genetically abrogated the pentose phosphate pathway (PPP), and suppressed the glycolysis pathway. This also attenuated the succinate pathway and readdressed the whole pyruvate flux to succinate. By following these modifications, they successfully hiked HA titers to 28.7 g L^{-1} from 1.3 g L^{-1}. A metabolic engineering strategy of co-substrate utilization was applied to establish galactose conversion into UDP-glucuronic acid, which is an essential precursor molecule for HA biosynthesis in an engineered *E. coli* strain (Woo

et al., 2019). They eliminated repressor-encoding genes by knocking out the *galR* and *galS* genes responsible for checking the galactose-utilizing Leloir pathway. The hasA gene from *Streptococcus zooepidemicus* was inserted to express the HA production pathway. The removal of the pfkA and zwf genes which respectively encode 6-phosphofructokinase I and glucose-6-phosphate dehydrogenase, affected the rates at which glucose and galactose were consumed. Additionally, by selective upregulation of the gene clusters such as *galU-ugd* and *glmS-glmM-glmU*, which promote the formation of UDP-glucuronic acid and UDP-GlcNAc, they altered the precursor biosynthesis pathway for the creation of HA. Using co-substrates glucose and galactose, the batch culture of the ultimate recombinant strain produced ~30g L^{-1} HA with 3.61-fold higher titers than that of the wild type strain.

Another group has also developed novel *B. subtilis* strain for HA production using metabolic engineering. Since, the generation of heterologous HA puts cell wall synthesis and central metabolism in direct competition. Thus, they stated that suppressing pfkA and/or zwf expressions may be a useful strategy to divert carbon flux away from glycolysis and the PPP, respectively, and towards cell wall biosynthesis. Eventually, HA biosynthesis, showed least amount of negative effects on cell physiology (Westbrook et al., 2018). Frequently adopted approach for heterologous HA production is co-express the core biosynthetic enzymes such as HasA (hyaluronan synthase; HAS), HasB (dehydrogenase), HasC (uridyltransferase), HasD (acetyltransferase) and HasE (phosphoglucoisomerase). Using *C. glutamicum*, the ideal operon arrangement for HA production (HasAB and HasABC were found to be optimal for HA biosynthesis in *C. glutamicum*) and examined how the lactate dehydrogenase (LDH) gene deletion by knocking out affected the build-up of HA (Cheng et al., 2017). In Batch and fed-batch fermentation in a 5-L fermenter with modified strain's and glucose feeding showed 21.6 g L^{-1}, HA titer which was more than 3-fold that of the wild-type *Streptococcus*.

Researchers used indirect pathway metabolic engineering approaches to boost HA production in engineered *C. glutamicum* in a separate study (Du et al., 2021). Their four parameters were the targets for modification separately. The carbon source is the primary factor for cell growth and glycan synthesis. There is a non-PTS (phosphotransferase system independent) pathway for glucose uptakes in *C. glutamicum*, called as the IPGS coupling system. In this system, glucose is entered inside the cells by myo-inositol permease (gene: IolT1/IolT2) and phosphorylated by glucokinases (Glk and PpgK). However, this pathway is suppressed by the GntT-type regulator

IolR. So, in the first strategy, they knock out the iolR gene, which activates the non-PTS system and the efficient uptake of glucose. HAS is important for HA synthesis. It has been reported that CL acts as a HAS inducer in *S. equisimilis*. CL is synthesized by the key enzyme phosphatidylglycerol phosphate synthase, which is encoded by two copies of *C. glutamicum* pgsA1 and pgsA2, as well as the cls (Ruan et al., 2020). In the second strategy, pgsA1 and pgsA2 were overexpressed, as were the CLS genes, indirectly increasing HAS efficiency and HA production. The VHb gene, present in the obligate aerobic bacteria *Vitreoscilla*, can be expressed in two different ways, improving cell respiration and energy consumption even when oxygen is scarce and, eventually, boosting the production of HA. They identified the essential role of glutamine in HA synthesis via transcriptome analysis. Subsequently, supplementing production media with glutamine improved HA titers. Low molecular weight heparosan was transferred and cloned into three non-pathogenic *E. coli* strains: BL21 (DE3), HT115 (DE3), and Shuffle T7 (DE3), by carefully analyzing each host bacteria's growth parameters, such as culture media composition, culture times, and cloning orders. The research team regulated the heparosan chain length range from 150 KDa to 5 KDa (Roy et al., 2021). Thereby, they successfully engineered *E. coli* and enhanced the low-molecular-weight heparosan polymer production using a one-step process.

The HA biosynthesis pathway was established in *B. amyloliquefaciens* by introducing the HA and UDP-GlcUA synthase genes (Ma et al., 2022). Second, the production of HA was increased by 13% after the inhibition of polysaccharide production through the knockout of related genes such as sacB (which encodes levansucrase) and epsA-O (which encodes extracellular polysaccharides) using the clustered regularly interspaced palindromic repeats (CRISPR/Cas9n) system. After adjusting the fermentation conditions and adding osmotic agents, 2.89 g L^{-1} of HA with a high molecular weight of 1.5 MDa was successfully produced. The results concluded that HA can be produced sustainably in engineered *B. amyloliquefaciens*.

3. Different target sites in bacteria for glycol-engineering

3.1. Pathway targeting to capsule polysaccharides

Capsular polysaccharides (CPSs) are complex carbohydrates that surround the cell wall of many bacteria, providing protection against the immune system and contributing to virulence (Willis and Whitfield, 2013). Importantly, they can be modified either chemically or through metabolic

engineering. Metabolic engineering of CPSs has numerous applications, including the development of new vaccines and therapeutics, as well as the production of high-value polysaccharides for use in the food and cosmetic industries. Additionally, due to the intrinsic advantage of avoiding potential contamination issues, using the CPS's recombination system is favourable (Chae et al., 2017; Cho et al., 2015). Moreover, CPS yield can effectively be enhanced by process optimization, as CPS production can strongly depend on physical parameters including temperature, pH value, culture medium components, and methods of fermentation cultivation such as feeding strategies (Wang et al., 2010).

A comprehensive knowledge of the biosynthesis processes is required for CPS production using a metabolic engineering approach. Generally, capsular genes that participate in bacterial CPS synthesis are organized in clusters in the genomes of those particular organisms. In *E. coli*, capsular genes are clustered into three regions. Gene correspondence to Region 2 (the central region) is specific for CPS polymerization. Additionally, these border genes are conserved across multiple serotypes and code for diagnostic marker proteins for biosynthetic systems. With group 2 capsules, various strains of *E. coli* can flip between Region 1 and Region 3 genes. Most significantly, these genes are capable of transfer, have a separate role in the synthesis of CPS structures, and are employed in a number of processes such as export and capsule construction on the cell surface (Cress et al., 2014). Two UDP-sugar intermediates are used for the biosynthesis of the GAG-like CPSs, and only one of them utilizes for CPS synthesis. Precursors of UDP-sugar are alternately connected to the non-reducing end of the developing polysaccharide chain during the biosynthesis process (Williams et al., 2018). This pathway is a representation of many other bacteria (Figure 18A,B).

E. coli K4 and type F *Pasteurella multocida* produce CPSs, which are related to CS. Whereas, *E. coli K4* naturally produces a CPS with a disaccharide repeat unit similar to fructosylated chondroitin, offering a less expensive and secure substitute for chondroitin derived from animals via bacteria fermentation (Vaidyanathan et al., 2017). In their independent and consecutive studies, homologous overexpression of chondroitin polymerase (the kfoC gene), mediated by the IS2 transposon, and homologous overexpression of the rfaH gene (a positive regulator that controls K4 expression of the polysaccharide biosynthesis genes) in *E. coli* K4 was reported that shows substantial increased CS productivity (Cimini et al., 2013). Another team that used metabolic glycoengineering overexpressed the transcriptional regulator SlyA, which led to

an increase in the expression of the K4 capsule gene cluster and a faster rate of fructosylated chondroitin production (Wu et al., 2013).

Increased expression of genes *pgm* and *galU* (involved in biosynthesis of biochemical precursor for UDP-GlcA) by introducing additional copies showed substantial increased in CPSs polysaccharide concentrations in *E. coli* K4 (Cimini et al., 2015). Additions of precursor supplements to growth medium also improve CPS yields. Increasing UDP-GalNac byosynthesis by using glutamine-enriched medium significantly enhances CPS yields (Xu et al., 2012). Similarly, researchers found that a lower amount of GlcA in the growth medium showed an imbalance in the UDP-GalNAc and UDP-GlcA ratios in *E. coli* K4 (Restaino et al., 2013). This problem was rectified by adding GlcA and GalNAc monosaccharides to the growth medium of K4 at the starting point, which led to a 64% increase CPS production (Restaino et al., 2017).

A native heparosan CPS producer, *E. coli* K5 growth-associated CPS heparosan, was enhanced by fermentation process optimization (Wang et al., 2011). It was identified that heparosan chain length has been dependable on fermentation conditions, which can further be controlled by manipulating the type 3 synthase gene and medium substrate concentrations like UDP-sugar (Ventura et al., 2006). Later, other *E. coli* hosts were engineered to resolve the native *E. coli* K5 pathogenicity problem. Researchers used plasmid transformation methods and transferred four *E. coli* K5 heparosan byosynthesis genes (*kfiA, kfiB, kfiC,* and *kfiD)* into *E. coli* BL.21 (Zhang et al., 2012). The modified strain is able to yield 334 mg/l of heparosan. When the *kfiC* gene was fused with the trigger factor tig gene of *E. coli* to stabilize *kfiC,* the resulting yield was further improved to 1.5 g/l in the engineered BL.21 strain (Leroux and Priem, 2016).

Polysialic acid (PSA) is a linear polymer of sialic acid that can be synthesized by many bacteria. Metabolic engineering was used to modify the *E. coli* SA8 strain by overexpressing two PSA biosynthetic pathway enzymes, NeuD and NeuA. Subsequently, they blocked the competing catabolic pathway product Neu5Ac by deleting the nanA gene responsible for expressing Neu5Ac aldose, a lyase involved in Neu5Ac synthesis. The final modified strain produced 85% more PSA with a final titer of 16.15 g L^{-1} in fed-batch fermentation (Chen et al., 2015).

3.2. Metabolic engineering of eukaryotic polysaccharides on the lipid anchored Und-PP bacteria

Because it is not always feasible or preferred to directly polymerize the LPS core. As a result, researchers have looked for additional sites for polysaccharide conjugation, such as the typical lipid anchor, Und-PP. The ECA and O-antigen manufacturing routes in *E. coli* K-12 entail the installation of a GlcNAc residue on Und-PP by an initial glycosyltransferase named WecA. A tetrasaccharide mimicking the LeX antigen (without the fucose residue) was built on Und-PP by adding glycosyltransferases from *Haemophilus influenza.* Lipo-oligosaccharide (LOS) biosynthesis pathway that was capable of altering this Und-PP-linked GlcNAc in the recombinant system. The use of this lipid as a carrier allowed the glycan to be conjugated to a protein via an oligosaccharyltransferase-mediated channel. The purified glycoconjugate has undergone in vitro enzymatic elaboration to add the fucose residue to complete the LeX structure (Hug et al., 2011). The use of modified bacteria to manufacture LeX-containing glycoproteins is noteworthy since these proteins are known to operate as immunomodulatory molecules (Atochina et al., 2001). Its efficacy has been proven in animal models to alleviate symptoms associated with autoimmune illnesses (Atochina and Harn, 2006). The Thomsen-Friedrich antigen (T antigen), a Gal1-3GalNAc disaccharide, is another human-like glycan generated in a similar fashion. Valentine and their research team created the T antigen disaccharide using UndPP-linked GlcNAc as a primer (Valentine et al., 2016). To assure the availability of the necessary substrate UDP-GalNAc, two heterologous glycosyltransferases and a nucleotide sugar epimerase were added. Because T antigen expression is elevated in many cancers, including prostate, breast, colon, and stomach tumors, recombinant biosynthesis might provide extremely immunogenic glycoconjugates that trigger antibodies against this critical glycan epitope (Heimburg-Molinaro et al., 2011). In a bottom-up engineering approach, the conserved core of trimannosyl chitobiose glycans (Man3GlcNAc2) in all humans was successfully generated on Und-PP by co-expression of 4 eukaryotic glycosyltransferases, the yeast uridine diphosphate-GlcNAc transferases Alg13 and Alg14, and the mannosyltransferases Alg1 and Alg2 (Heimburg-Molinaro et al., 2011). The Man3GlcNAc2 structure has been proven to be the minimum structure necessary for glycoprotein therapeutics' effectiveness, and it is the most often conjugated glycoform to proteins produced in a baculovirus host system. Furthermore, because this structure represents the conserved core of human N-glycans, it has great potential as a precursor for further alteration, either in vivo or in vitro (Van Patten et al., 2007).

3.3. Metabolic engineering of eukaryotic polysaccharides on bacteria LPS

Gram-negative bacteria are surrounded by a dense LPS layer embedded in an outer membrane. Functionally, LPS delivers structural integrity to the bacterial cell and is the major component that determines the bacteria's pathogenicity. As a result, LPS appeared to be an excellent target for metabolic glycan engineering in Gram-negative bacteria via specific and well-defined metabolic labeling of glycans. In Gram-negative bacteria, LPS is composed of membrane-anchored lipid A with a core oligosacchride attached to it and o-antigenic polysacchride extending outwards from the bacterial cell (O-antigen-determined species variation). The respective genes involved, and their functions. Wzx translocates O-antigen subunits across the inner membrane, while Wzy polymerizes the chain up to the length determined by Wzz, and WaaL helps in the ligation of the chain onto complete Core-Lipid A molecules, which are separately translocated by MsbA (Wang et al., 2010). Then LPS molecules are assembled and translocated across the peptidoglycan and outer membrane by the proteins LptA, B, C, D, E, F, and G (Ruiz et al., 2009). The inner core of LPS is generally composed of two or three Kdo units, whereas the outer core is made up of the most common monosaccharides, such as glucose and galactose (Figure 19).

Researchers also attempted to tag the *E. coli* LPS O-antigen with azido labeling of a 3-deoxy-d-mannooctulosonic acid (KDO), a specific and necessary component of LPS's inner core, and at least one of its residues is directly connected to lipid A (Edens, 2005). Due to the vital importance of KDO for Gram-negative bacteria, its pathway is considered a potential target for novel antibiotics. The modified image was then used to image the bacteria. Interestingly, Gram-positive strains did not exploit KDO in their cell walls; thus engineering KDO offers a way to image or deliver drugs to concerned bacteria (Dumont et al., 2012). According to the study, the insertion of bioorthogonal handles into LPS structures offers a promising technological platform for identifying clinically relevant areas that might be targets for medication administration and imaging bacteria for early infection identification. Humans with Sigha toxin-producing *E. coli* (STEC) infections have post-diarrhoeal HUS, a potentially fatal illness that causes hemolytic anaemia and renal failure. Stool culture and O157:H7 serotype detection are both part of the STEC diagnosis. Recombinant glycoproteins with the polysaccharides O157, O145, and O121 coupled with carrier proteins were created in a separate study using metabolic glycoengineering technology. These glycoproteins serve as serogroup-specific antigens employed in the serological diagnosis of STEC-associated HUS. When these antigens were tested in indirect ELISAs (glycol-

iELISAs), the results showed that STEC O121, O145, and O157 could be distinguished between healthy and infected children. In addition, Shiga-toxin (Stx), a strong AB5 toxin generated by *Shigella* dysenteriae and *E. coli* O157, binds to the human glycosphingolipid globotriaosylceramide (Gb3) receptor. This receptor scaffold contains the trisaccharide, Gal(b1-4)Gal(b1-4)Glc is found in several eukaryotic cell types and is abundantly found in renal tissue and microvascular endothelial cells (Paton et al., 2000).

Neisseria spp. produces a similar structure of the Gb3 receptor as a LOS component as a tactic of host immune envasion (Cress et al., 2014). The glycosyltransferases LtxC from *N. meningitidis* and LtxE from *N. gonorrhoeae* were expressed in *E. coli*, resulting in the formation of a new LPS-associated Gb3 polysaccharide scaffold. Paton AW and co-workers expressed LtxC of *N. meningitidi*s and LtxE of *N. gonorrhoeae* into *E. coli* and reported the production of a novel LPS-associated Gb3 polysaccharide scaffold. Further, administering engineered *E. coli* to mice suggested an effective molecular mimic of the toxin binding site (Paton et al., 2000). Similarly, another research group expressed globotetraosylceramide (Gb4) and the gangliosides GM_1 and GM_2 in *E. coli*, which have also proven effective against cholera and STEC in animal models (Fischöder et al., 2019).

4. Tools and strategies for the synthesis of complex and conjugated glycans

In order to create microbial cell factories more quickly and efficiently, systems metabolic engineering was recently developed by combining conventional metabolic engineering techniques with those of synthetic biology, systems biology, and evolutionary engineering. Systems metabolic engineering (SME) considers multiple processes simultaneously while developing new microbial strains, including upstream activities related to raw material preparation, midstream processes related to fermentation, and downstream processes related to recovery and purification (Ko et al., 2020). Incorporating systems biology methods and strategies enables genome-wide-scale estimation of cellular conditions, including metabolic fluxes. For a wider spectrum of substances and materials, production routes and enzymes can be designed and constructed using synthetic biology approaches. SME allows for the creation of advanced microbial cell factories that can efficiently produce a variety of glycans.

4.1. Metabolic engineering of regulatory network

On the chromosome, regulatory genes for particular metabolic pathways are frequently found in clusters with genes for the biosynthesis of those pathways. Pathway specific regulators can influence the expression of gene cluster elements in either a positive (activators) or negative (repressors) way. In most bacteria, not all of the genes are expressed at once. Although microbes are excellent at providing us with a wide range of important products, they often only make them in the quantities that they require for their own survival; as a result, they don't frequently overproduce their metabolites (Kumar and Prasad, 2011). However, we want industries to continuously synthesize items. For that, we need to engineer microorganisms using an up-and-down regulatory strategy.

Studies to improve complicated features, such as global transcription machinery engineering (gTME), have been further encouraged by the engineering of regulator proteins using random mutagenesis and directed evolution approaches. It is possible to use mutations in transcriptional regulators to change cellular metabolism and improve specific phenotypes. The number of genes whose expression is altered by these methods is constrained. In order to engineer complicated phenotypes, a technique is therefore required to integrate all of the interactions among regulators and create a thorough and sensitive regulatory network (Tan et al., 2016).

A regulatory network is composed of regulators that collaborate with genes and proteins to regulate the number of mRNAs and proteins expressed at any given time. More than 4000 genes in *E. coli* are controlled by more than 200 regulators (Salgado et al., 2006). Thus, by changing regulatory networks, a highly diversified mutational library directed to the active regions of several regulators might easily interfere with cellular metabolism (Liu, 2015). CRISPR-Cas techniques are currently used for targeted mutagenesis at the genome scale (Si et al., 2017). In the field of synthetic biology and genome engineering, these CRISPR-based techniques have great editing efficiency and facilitate a wide range of mutations (such as insertions, deletions, and point mutations), allowing us to more widely and thoroughly investigate the genetic forum and improve our comprehension of biological systems. Recently, Liu and co-workers located an overall regulatory network of regulatory genes in *E. coli* that controls or communicates with thousands of other genes (Liu et al., 2020). They created a large-scale saturation mutagenesis library that specifically targets the active sites of regulators in the network using a CRISPR-based technique that was previously established, called CRISPR-enabled trackable genome engineering (Garst et al., 2017). They screen and select many complicated features necessary for industrial applications

using this regulatory network library. Using this approach, the team was able to screen reported and previously identified mutations that enhanced tolerance power and improved production phenotypes compared to previous studies.

4.2. Rational engineering of metabolic flux

Rational engineering of metabolic flux refers to the deliberate design and manipulation of metabolic pathways in living organisms to optimize the production of specific desired compounds or improve overall metabolic performance. This can be achieved through genetic modifications or other engineering strategies to enhance the efficiency of metabolic pathways, redirect metabolic flux towards desired products, and improve the overall yield and productivity of a particular metabolic process. Rational engineering of metabolic flux has wide-ranging applications in various fields such as biotechnology, bioengineering, and synthetic biology, and has the potential to revolutionize the production of biofuels, pharmaceuticals, industrial chemicals, and other valuable compounds. For native producers, the introduction of the salvage route can result in the development of a twofold pathway, improving the production of nucleotide sugars. This method is frequently used to create HMOs, particularly fucosylated and sialylated HMOs. For example, GDPFuc is naturally produced by *E. coli* using an inherent de novo pathway (Petschacher and Nidetzky, 2016). Therefore, the introduction of the salvage pathway, which contains L-fucokinase/GDP-L-fucose phosphorylase (FKP), triggered an increase in GDP-Fuc for fucosyllactose (FL) production employing Fuc as a substrate.

In order to improve the precursor pools, limiting the competing pathways is a widely utilized method of controlling the dispersion of metabolic flux. GDP-fucose is a primary sugar donor for the manufacture of fucosyllactose as well as a crucial substrate for the synthesis of colanic acid. GDP-fucose production in *E. coli* was increased by knocking down the UDP-glucose lipid carrier transferase gene wcaJ, which controls colanic acid biosynthesis. Subsequently, a final 2'- FL (2'-fucosyllactose) titer of 11 g L^{-1} was obtained by further overexpression of the rcsA gene, an up-regulator of de novo GDP-fucose production (Drouillard et al., 2006). When competing pathways are deleted, monosaccharides become one of the main carbon sources for cellular growth and get converted in the salvage pathway by many isomerases instead of being utilized in neucleotide sugar synthesis. Therefore, simultaneous deletion of isomerases along with competing pathways drives more sugar flow to precursor formation (Jung et al., 2019).

Although the above two metabolic fluxes promote sugar donor biosynthesis, bacteria have some absorbtion limits for substrate. This is the rate-limiting step in the synthesis of oligo and polysaccharides in a cell factory. Substrate import can be increased effectively by deactivating the hydrolase and adding transport proteins. These methods are frequently employed in the production of HMOs, which use lactose as an acceptor for glycosyl transfer. Galactosidase (*LacZ*) was eliminated or only partially inactivated to control lactose hydrolysis, and a redesigned lactose operon (*LacY*) was added to the cell to boost lactose absorption (Choi et al., 2019; Huang et al., 2017). Similarly, Neu5Ac transporter *NanT* expression was introduced and sialic acid aldolase (*NanA*) gene was knockout to enhanced extracellular Neu5Ac and prevent degradation for sialylated HMO sialylactose synthesis (Priem et al., 2002).

Using living cell factories for oligo/polysaccharrides productions energy required in form of ATP. Natively, pyruvic acid metabolizes through tricarboxylic acid (TCA) cycle to sustain cell growth and metabolite production. In contrast, lactate dehydrogenase (LDH) catalyzes the conversion of pyruvic acid to lactic acid, thus act as limiting reaction for metabolite production. Therefore blocking the lactic acid production pathway via deleting LDH expressing genes leading to increase availability of ATP (Zhang et al., 2022).

4.3. Over expression and modification of key enzymes

Continuing with the construction of competent metabolic flux pathways and competing pathway gene removal, altering pivotal genes responsible for producing key enzymes by overexpressing them is considered a direct and effective way to regulate oligo/polysaccharide production. The PPP and TCA cycle enzymes such as isocitrate dehydrogenase, membrane-bound transhydrogenase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase are important for NADPH regeneration. Therefore, their genes are overexpressed using metabolic engineering in order to induce a severely reduced state in E. coli and increase the production of fucosyllactose (Huang et al., 2017). Similarly, modifications in genes to overexpress chontroitin synthase for chondroitin production, HA synthase for hyaluronan, and α-1,2/1,3 fucosyltransferase for fucosylated HMOs have been experimented with to enhance target oligo/polysaccharide prodduction (Engels and Elling, 2014; Zhang et al., 2016). However, limitations of this strategy include the burden on cells for carbon utilisation and metabolism. Thus, a precise, well-tuned modulation of the pathway, such as optimization of the ribosome-binding site and identifying the

tunable intergenic regions that maximize and maintain oligo/polysaccharide production along with cell growth.

Such a modulated pathway design would work systematically following precursor accumulations, then polymerization, leading to high target carbohydrate producion (Zhang et al., 2018). Cimini and colleagues improved the polymerization module of the pathway by overexpressing the chondroitin polymerase KfoC gene. Subsequently, precursor sugar donors UDP-GalNAc and UDP-GlcA generation was enhanced via modulating genes for overexpressing biosynthetic enzymes, simultaneously blocking metabolic flux from F-6-P to fructose-1,6-bisphosphate, resulting in optimization of the precursor module (Cimini et al., 2015). Overexpression of fusion proteins assists in forming interactions between catalyzing enzymes in sequential pathways and substrates. Insertion of such fusion protein operons is also a common tactic to prevent important metabolites diffusion and consumption by competitive enzymes (Liu et al., 2013). For example, overexpression of the hasA-B-C operon in recombinant *Lactococcus* lactis enhanced HA production to 1.8 g L^{-1}. It was reported that varied combinations of operons result in different amounts of titer (Prasad et al., 2010). Cheng and coworkers engineered *C. glutamicum* and developed eight strains with different operons (Cheng et al., 2017). On comparing strains for HA production, it was confirmed that hasA-B was the best operon for HA synthesis with a titer of 21.6 g L^{-1} compared to operons hasA-D, hasA-C, and hasA-E, which synthesized low HA titers in similar ways to single hasA strains.

4.4. Selection of host strains for heterologous pathway insertion

To enable the successful production of targeted chemicals, trustworthy microbial host strain selection is crucial. Because of our detailed knowledge of their metabolisms and the availability of gene and genome editing technologies, *E. coli* and *S. cerevisiae* have been selected as the two most prominent host strains among a variety of microorganisms (Lee et al., 2019). Due to recent developments in modern engineering tools that allow the effective manipulation of various microorganisms, including those less explored, we are no longer restricted to using *E. coli* for the production of tailored glycans and can use microorganisms with inherently superior metabolic characteristics towards the target products. Any pathogenic microorganisms shouldn't be employed as production hosts; that should go without saying. Consider whether the microbe naturally produces too much of the desired product, does so inefficiently, or does not create the

desired product at all when choosing the production host. A variety of metabolic glycan engineering strategies are applied to each of these three conditions.

Natural overproducers are undoubtedly the preferable host strains for the effective manufacture of glycans because they have already grown to have a great tolerance and sturdy metabolic fluxes for the desired products. For example, the human pathogen *C. jejuni* has an N-glycosylation mechanism that is the best studied (Szymanski et al., 1999). The Glc GalNAc5 Bac structure of the *C. jejuni* glycan is a branched heptasaccharide, where Bac is for bacillosamine. A range of periplasmic and extracellular glycoproteins, such as antibodies (Fisher et al., 2011) and conjugate vaccines (Feldman et al., 2005), have been created using *E. coli* cells that are glycosylation-competent.

E. coli as a host expression system has shown considerable promise as a platform for glycoprotein production due to significant breakthroughs and unmet demands. Effective prokaryotic glycosylation currently needs a bacterial strain with a DwaaL genotype, as shown by the frequently used *E. coli* W3110 mutant, CLM24 (Jaffe et al., 2014). WecA, a natural *E. coli* protein, was found due to mutational research to determine the task of the proteins involved in the *C. jejuni* protein glycosylation (Pgl) construct. It can transfer GlcNAc, the first eukaryotic core sugar, to the lipid-linked precursor, which serves as the foundation for the remaining bacterial glycan (Linton et al., 2005). After removing the genes that produce and a peptide-proximal 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH), the rare sugar that is the pioneer residue on the *C. jejuni* glycan, WecA was able to function by introducing GlcNAc as the primary sugar (Schwarz et al., 2010).

A strain of *E. coli* that can produce O-glycosylated proteins in vivo has been created (Du et al., 2019). To do this, a twin plasmid method was adopted, with one encoding a target therapeutic protein and the other the O-glycosylation-related enzyme machinery. The latter plasmid contains the genes for a human polypeptide N-acetylgalactosaminyl transferase, a 1,3-galactosyl transferase, a *C. jejuni* UDP-Glc(NAc)-4-epimerase, and a bacterial or human disulfide bond isomerase. The efficacy of this two-plasmid synthetic operon system has been evaluated using native and engineered forms of naturally O-glycosylated human interferon-2b and human growth hormone with one altered site of glycosylation, three proteins with therapeutic promise. In a study, a terminally sialylated N-glycoprotein that resembles the sialylated moiety (Neu5Ac-α-2,6-Gal-β-

1,4-GlcNAc-) of human glycans can be produced through an *E. coli* periplasmic pathway (Zhu et al., 2020).

E. coli metabolic engineering has produced a wide range of free oligosaccharides for both academic and industrial use. The next significant problem is the site-specific synthesis of oligosaccharides on proteins in the bacterial cytoplasm, which is now being prepared for by researchers. The efforts centre on the metabolic glycoengineering of an N-glycosylation pathway in the cytoplasm of *E. coli,* utilizing heterologous glycosyltransferases to manufacture specific glycoprotein structures. The N-X-S/T sequon is the starting point for the recently identified family of cytosolic N-glycosyltransferases, which allocate a single glucose residue onto proteins at asparagine (N) residues (Valderrama-Rincon et al., 2012). It has been established that N-linked lactose is produced in the *E. coli* cytoplasm when N-glycosyltransferases and a galactosyltransferase are co-expressed. The design of cytosolic N-glycosylation mechanisms to synthesize significant glycans on proteins can start with this N-linked lactose. It is important to test bacterial glycosyltransferases for their capacity to extend N-lactose with specific sugars, resulting in a wide range of glycans that are synthesized directly on proteins. In order to effectively produce fucosylated oligosaccharides, which are essential glycan epitopes in a variety of physiological processes, the focus must be on identifying fucosyltransferases. Various protein substrates should be used with the recently created glycosylation pathways to investigate and solve any potential flaws in the glycosylation system. The result may be a well-defined toolkit for glycoengineering that allows *E. coli* to produce specific glycoprotein structures from the bottom up.

4.5. High-throughput screening tools

The required mutant can be easily obtained using a variety of methods such as FACS, colorimetric assays, spectrophotometers, or microfluidic sorting apparatuses. With enzyme assays or strain screening, it is possible to screen for alterations in colour, fluorescence, size, higher cell density, or other simply noticeable features. However, many enzymes and strains lack such readily screenable features. The lack of appropriate screening techniques to choose superior variations from a population of strains or enzymes in such circumstances is a severe bottleneck in evolutionary engineering. Thus, applying slow and low-throughput methods like HPLC, GC, or mass spectrometry frequently restricts the choice of an enzyme or strain with the required

properties. Synthetic biology techniques can solve this problem when there are no simple screening methods available by creating novel screening platforms that broaden the range of features that could be selected and screened.

For high-throughput screening applications, biosensors that could detect cellular metabolism in vivo are attractive. To quickly test for the desired feature, high-throughput screening techniques like FACS may be paired with genetically programmed biosensors that interact with intracellular metabolites and produce legible output. Syntheses of glycans involve numerous enzyme reactions, and the level of reaction is characterized by the concentration of reaction products, intermediate substances, or some energy molecules. Theoretically, high-throughput screening and detection of polysaccharide production levels within a single cell can be accomplished using biosensors that detect these intermediate substrates or products (Li et al., 2020; Rogers and Church, 2016). An actuator part (such as fluorescent reporters, regulatory switches, or selection markers) is included in biosensors along with a sensor part (such as riboswitches, enzymes, ribozymes, transcription factors, or periplasmic-binding proteins) (Michener et al., 2012). Biosensors for polysaccharide synthesis target substances involved in glycan chain synthesis and energy metabolism. For example, during hyaluronic acid production, cells consume five ATP molecules, two NADH molecules, and one acetyl-CoA.

In carbohydrate metabolism, pyruvate transformation involves acetyl-CoA and tricarboxylic acid. Exploiting this Förster Resonance Energy Transfer (FRET) sensor, this detected the pyruvate concentration in real time. The FRET biosensor demonstrated excellent orthogonality, high time resolution, and a simple construction method. Similarly, a NADPH/$NADP^+$ redox-detecting biosensor was developed for *E. coli* that used the natural redox-sensitive transcriptional factor (TF) SoxR to detect and sort mutant Lactobacillus brevis (LbAdh) strains for NADPH-dependent alcohol dehydrogenase, showing enhanced activity for substrate 4-methyl-2-pentanone (Siedler et al., 2014). A monosaccharide-responsive bisensor has been developed based on the high specificity and affinity of Neu5Ac aptazymes that monitor real-time concentrations of Neu5Ac (Cho et al., 2013). For the first time, an in vivo active biosensor was created that responds to NeuAc in a dose-dependent manner (Yang et al., 2017). This is an advanced evolutionary Neu5Ac aptazyme-based biosensor in which the gene tetA (which encodes a tetracycline/H^+ antiporter, confers the cell's tetracycline resistance, and renders the cell sensitised to the toxic metal $NiCl_2$) was fused to the NeuAc aptazyme. Under selection circumstances,

intracellular Neu5Ac content was directly related to cell growth as the Neu5Ac biosensor detected those mutant cells that accumulated a high concentration of Neu5Ac, which helped the cells survive in high $NiCl_2$ conditions. Since biosensors enable high-throughput mutation screening, the author reported an increased Neu5Ac titer from 1.58 g L^{-1} to 2.61 g L^{-1}.

5. Biopharmaceutical applications of bacterial engineered glycans

Research and progress in the area of metabolic engineering have established this technology's versatile application to biopharmaceutics and advanced healthcare on several fronts. By manipulating some targeted glycans displayed on the cell surface using metabolic engineering, it is easy to modulate inside-cell behaviour. The production of tailored glycans with upgraded pharmacokinetics and additional new delivery strategies for in vivo tests impacts the promising role of metabolic engineering for clinical translation. In this section, we discussed several aspects of tailored glycan products derived via metabolic engineering techniques in anticipation of recent applications to human healthcare (Table 2).

5.1. Metabolic glycoengineering emerged as a novel antibacterial strategy

In bacterial glycoconjugates synthesized through glycosylation reactions, the glycan product incorporates some unique monosaccharides such as 2,4-diacetamido-2,4,6-trideoxygalactose, N-acetylfucosamine, bacillosamine, 3-deoxy-d-manno-octulosonic acid, legionaminic acid, rhamnose, and others. Studies report that bacterial pathogenicity is attributed to a variety of virulence factors produced by any strain. Also, many bacterial virulence factors, such as glycoproteins, oligo- and polysaccharides, and glycotransferase effector proteins, are the products of biosynthetic glycosylation pathways. These glycans contain monosaccharides with species-specific features and contribute to forming unique glycan structures, making them suitable targets for antivirulence therapies (Williams et al., 2020).

Bacterial flagella play an important role in infection spreading, and many flagella glycans are attributed to bacteria-specific monosaccharides that impart their characteristic diverse structures. For instance, Pse and Leg, along with derivatives of methylglycerol and acetamido moieties, are essential for the proper flagellar assembly process and are catalyzed consecutively by the enzymes PseB-PseC-PseH-PseG-PseI. It was reported that strains of *H. pylori* and *C. jejuni* with non-operating Pse gene expression showed defective flagella. Three small molecules were discovered that inhibited the PseB enzyme of the pseudaminic acid biosynthetic pathway, thereby

inhibiting flagellin protein production in *C. jejuni*. This bacterium also incorporates the Pse derivative 7-acetamidino-Pse (Pse5Ac7Am) during O-linked flagellin glycosylation (Howard et al., 2009). Interestingly, other researchers identified that the phage protein FlaGrab precisely binds to Pse5Ac7Am and modified flagellins in *C. jejuni*, which causes low motility and partial growth reduction (Sacher et al., 2020). GT1 (core GlcNAc transfer onto Ser/Thr), GT2 (Rha transfer and Rha methylation), and GT3 are the glycosyltransferases necessary for the production of the type B glycan. Here, GT1 and GT2 from the *Clostridioides difficile* strain were successfully knocked out, resulting in low motility in the bacterium. Moreover, the mutant strains showed reduced adherence efficiency (Valiente et al., 2016). This study suggested the importance of MGE in type B glycan production against hypervirulent strains of *C. difficile*.

Metabolic engineering is used to boost the cytosolic supply of NADPH via the PPP. *Streptomyces lividans* TK24 was engineered for carbon distribution and NADPH production by overexpressing the NADPH-producing enzymes glucose 6-phosphate dehydrogenase (genes: *zwf1* and *zwf2*) and 6-phosphogluconate dehydrogenase (gene: *zwf3*). Furthermore, one allosteric effector gene, opc, which is required for ZWF activity, was found to be overexpressed in various combinations. The final recombinant strain, *S. lividans* TK24/pWHM3-Z23O2, showed improved NADPH production and suggested that metabolic engineering of PPP routes is one of the suitable pathways for secondary metabolite production like antibiotics (Jin et al., 2017).

5.2. Metabolic glycol-engineering for bioconjugate vaccine production

Currently, metabolic glycoengineering utilized two broad bacterial glycosylation approaches: OTase-dependent or OTase-independent (taking place in the periplasmic space); both pathways exploit the bioconjugation of saccharides and proteins. Vaccines made with pathogenic bacteria's capsular polysaccharides as antigens typically do not activate T-cells and do not activate B-cells, making them ineffective in the long run. This weak antigenic response can be improved by incorporating target polysaccharides into a protein via bioconjugation.

Previously reported methods for bioconjugate vaccine synthesis included modification of bacterial polysaccharides (extracted from the target bacterium) and, after chemical modification, reinserting and linking them to the carrier protein. These conjugated vaccines are subjected to some downsides; the carbohydrates utilized for formulating such vaccines are generated from harmful pathogenic organisms that may be hazardous to health and require higher biosafety

precautions. Also, glycans with acid-liable sugars do not tolerate the chemical treatment steps requisite for purification and crosslinking to proteins. But in the past two decades, alternate strategies of glycoconjugate vaccine production in vivo in bacterial cells have yielded some potential vaccines closer to commercial licensure products. With the discovery of *C. jejuni* PgIB oligosaccharyltransferase, it becomes possible in glycoengineering to efficiently express glycosylated proteins, including capsular polysaccharides and lipoligosaccharides, in *E. coli* cells. Researchers expressed bacterial polysaccharides and glycoconjugated vaccines via metabolic engineering in a family of eleven *E. coli* strains (Kay et al., 2022). They transferred *S. pneumoniae* serotype 4 (SP4) capsule expression and glycans into *E. coli* using CRISPR recombination and developed rationally designed recombinant *E. coli* strains for the production of glycoconjugate vaccines. These newly developed strains were modified for reduced endotoxin toxicity by deleting the lpxM gene, produced more faithful glycan by deleting the wecA gene (transfer GlcNAc onto the lipid carrier), introduced undecaprenyl pyro glycan chain length determinants for *S. pneumonia*, and increased the glycan polymer length (Cognet et al., 2003). CjblB (OSTase) was also inserted into the genome in order to mitigate the metabolic load on the cell by minimizing the number of plasmids and antibiotics used for culture selectivity. All strains could potentially express *S. pneumoniae* capsular polysaccharides, increasing the yield of pneumococcal conjugate vaccines.

Using metabolically engineered *E. coli* host cells, a low-cost, one-of-a-kind recombinant pneumococcal protein-polysaccharide conjugate vaccine was created (Herbert et al., 2018). The vaccine was evaluated in a murine model of pneumococcal pneumonia, and its efficacy to prevent disease invasion was compared to that of Prevnar 13. The study found that glycoprotein conjugate vaccines can be successfully produced using metabolic glycol engineering techniques. A novel glycoconjugate vaccine was developed using a metabolic glycoengineering technique against *Shigella flexneri* serotype 2a (Kampf et al., 2015). Engineered PgIB transported the *S. flexneri* 2a O-polysaccharides to a non-virulent form of the *P. aeruginosa* carrier protein exotoxin A (EPA) (OTSase), leading to the production of glycosylated EPA-2a. Further in vivo production of this unique vaccine was optimized by carefully identifying and analyzing the glycoprotein synthesis process and parameters. They identified that consecutive induction of PgIB and the carrier protein EPA enhanced EPA-2a-specific productivity by a factor of 1.6. Additionally, during induction, EPA-2a production was further improved up to 3.1-fold upon introducing the monosaccharide N-

acetylglucosamine (10 g L^{-1}). They also identified the Mg^{2+} concentration (10 mM) for enzyme pathways. All optimized parameters were adjusted and incorporated into high cell density culture, which imparts a 46-fold increased yield of glycoconjugate compared to starting shake flask production.

Gram-negative bacteria produce OMVs primarily comprised of phospholipids, LPS, the outer membrane, and periplasmic proteins. Importantly, OMVs possess immunogenicity, self-adjuvant, and compatible intake by mammalian cells and can be modified by engineering, making them a good candidate for vaccine delivery. Researchers have developed strategies using metabolic glycol engineering for generating geOMVs displaying *S. pneumoniae* serotype 14 capsules for making conjugate vaccine in a non-pathogenic engineered strain of *E. coli* (Price et al., 2016). The recombinant strain was constructed in an *E. coli* strain lacking its O-antigen; they transferred the capsule synthesizing CPS locus, the required glycotransferases, the flippase (Wzx), and the polymerase (Wzy) needed for the synthesis of CPS14, and expressed them via an inducible plasmid sequence (pNLP80). CLM37 and CLM24, two distinct *E. coli* mutant strains, were used to assess the expression of CPS14. The enterobacterial common antigen and O-antigen initiator glycosyltransferase (WecA) synthesis initiator glycosyltransferase mutant strain CLM37. In the streptococcus capsule production process, WchA is the initial glycosyltransferase, which transfers a sugar residue to the Und-PP carrier. Both WecA and WchA compete for the common carrier for sugar-lipid attachments. As a result, interrupted WecA gene completion for Und-PP was reduced in CLM37, and CPS14 production was increased, whereas the CLM24 mutant was used to confirm CPS14 attachment to lipid A, as CLM24 is a waaL mutant in which glycans remain attached to the lipid carrier and no transfer of CPS14 to lipid A occurs. The resultant glycoconjugate vaccines (geOMVs) were examined against *S. pneumoniae* in mice and *C. jejuni* in chickens.

5.3. Other applications of metabolic engineering for tailored glycans production

According to research, N-glycan compositions have a major impact on how well IgG antibodies bind to immune cells' Fc receptors (FcRs) and C1q receptors, which in turn affect the activities of the antibodies. Fucose is a crucial component of glycoproteins, glycolipids, and other glycan structures. Previous research has shown that, when compared to fucosylated antibodies, antibodies without a fucose core have a substantially higher avidity for FcRIIIa (Ferrara et al., 2006), thus exerting enhanced antibody-dependent cell cytotoxicity (Mori et al., 2007). Therefore, engineering

Fc-glycans to eliminate core fucose is one of the great strategies to improve the IgG-FcR interaction. Researchers have identified a highly efficient recombinant fucosidases, namely BfFucH, from the CAZy database library of bacteria glycosidases present in *E. coli* (Tsai et al., 2017). The enzyme was able to hydrolyze the aforesaid fucosidic linkages, particularly the α-1,6-linkage present in the N-linked fucα-1,6-GlcNAc residue on glycoproteins. Subsequent study of BfFucH coupled with other fucosidase and endo-glycosidase showed α-1,6-linkage core fucose cleavage and transformed heterogeneous glycoproteins into homogenous glycoforms such as antibodies, Fc fusion proteins, interferon, and the influenza virus superficial glycoprotein hemagglutinin.

A crucial fundamental structural component of HMOs is LNT II. Due to its use in the creation of complex HMOs and its potential as a nutraceutical, LNT II is receiving a lot of attention. A metabolically modified *E. coli* was created that effectively produces LNT II consuming lactose as a substrate and glycerol as a carbon source (Zhu et al., 2021). The systematic modification of *E. coli* included strengthening the UDP-GlcNAc biosynthesis pathway and the incorporation of β-1,3-N-acetylglucosaminyltransferase (LgtA) to construct an LNT II-producing strain. Further, they made modifications at the ribosomal binding site to strengthen the transcription and translation rates of essential pathway enzymes and introduced multiple mutations to glucosamine-6-phosphate synthase (GlmS) to minimize feedback inhibition. Including the above-mentioned modifications, they constructed a number of engineered strains in which non-essential pathways were also removed by deleting their relevant genes. At last, culture conditions were optimized, and the resultant engineered strains produced 46.2 g L^{-1} of LNT II with a productivity of 0.77 g L-^{1} and a content of 0.95 g g^{-1} dry cell weight. According to recent research studies, several other HMOs with complex structures, including 2′-fucosyllactose, 3-fucosyllactose, lacto-N-neotetraose, 3′-sialyllactose, and 6′-sialyllactose, have been constructed via metabolic glycoengineering techniques (Choi et al., 2016; Dumon et al., 2004; Fierfort and Samain, 2008; Lee et al., 2011; Priem et al., 2002). Some important factors significantly influence recombination, such as host selection because of the uncertainty of metabolic patterns and the expression of appropriate glycosyltransferases in microbes.

Xylitol is a five-carbon sugar alcohol widely used in industries for various applications. It is used to make syrups, chewing gum, sweetening agents, and pharmaceutical drugs. Xylitol showed anti-cariogenic and anti-diabetic activities. Because it is in high demand, different methods

of producing it from the substrate glucose have been investigated, including fermentation and gene expression. In a single step of fermentation, the key enzyme called as xilitol-5-phosphate dehydrogenase (XPDH) converts glucose to xylitol. To improve xylitol production, researchers used a metabolic engineering approach to express the *xpdh* gene of *C. difficile* into an *E. coli* strain (using expression vector pACYC-Duet-1 and followed by induction by supplementing isopropyl -d-1-thiogalactopyranoside) with some metabolic pathway modifications (Abdullah et al., 2022). The subsequent modification of the recombinant strain was done by eliminating competing pathways that would yield four mutants: NA207 (DrpiA), NA208 (DrpiB), NA209 (Dpgi), and NA211 (DrpiApgi). Among them, mutant NA211 yielded the highest xylitol production, 0.585 g L^{-1}, and almost doubled the amount compared to the original strain.

The immune system of body generates antibodies against glycoproteins produced by cancerous cells, which are considered intriguing biomarkers for early cancer detection and could be analysed using glycoprotein diagnostics that exhibit cancer glycopeptides (Chatterjee et al., 2006). A glycopeptide array utilising synthetic O-glycosylated mucin segments was developed by adopting two approaches: chemoenzymatic fabrication of short glycopeptides and enzymatic creation of bigger mucin fusion proteins in *E. coli*, along with in vitro O-linked glycosylation utilising GalNAcTs. These studies demonstrate the possibilities and capability of utilising different glycoprotein production systems for use in diagnostic applications (Pedersen et al., 2011).

6. Future outlook and challenges

Since its discovery, metabolic engineering has extensively contributed to medicinal science, basic science, and the pharmaceutical industry. This newly emerging advanced future technology facilitates the efficient utilization of bacterial resources to generate tailored glycans of advanced biopharmaceutical and industrial importance (Martin, 2013). Furthermore, modern molecular methods like gene editing, deletion, and cloning have accentuated advancements in metabolic engineering. Genome-scale models designed for integrated metabolic flux, protein-glycan interaction, and identification of genes' expression regulatory networks are promoted in developing metabolic engineering strategies. However, detailed knowledge of the physiochemical properties of cells at the molecular level is required before implementing such techniques, including enzyme involvement in pathways with kinetics, metabolic flux distribution, and gene-protein interaction, among others, especially when metabolic systems are undergoing desired modifications.

In principle, metabolic engineering is implemented to efficiently produce the required materials, such as chemicals and biomolecules for drug discovery, healthcare, and food nutrition from heterologous microorganism hosts. The tools and techniques used to achieve the goals have focused on the overexpression and downregulation of certain genes to redistribute the steady-state fluxes of target metabolic pathways. Such required changes at the genomic level can be efficiently made through modern CRISPR-Cas9 technology. CRISPR-Cas9 offers a potential method for substantial precision over other gene editing technologies due to its simplicity of use, cost-effectiveness, and a relatively high degree of accuracy with efficiency (Roointan and Morowvat, 2016). Zinc-finger nucleases (ZFNs) are artificial restriction enzymes synthesized by combining a zinc-finger DNA-binding domain with a DNA-cleavage domain. It is a site-specific endonuclease that cleaves DNA at specific positions. ZFNs can be used to alter the genome of both lower and higher organisms precisely and can be used as a prominent tool for metabolic glycan engineering in bacteria (Ainley et al., 2013). TALEN, transcription activator-like effector nuclease technology, can also be utilized alongside CRISPR-Cas9 and ZFNs for genome manipulations (Joung and Sander, 2013).

In addition, site-specific recombinases offer excellent tools to manipulate pathway enzymes synthesizing genes on DNA segments. Another factor that influences the scale of production of tailored glycans is the design cycle (initial design, build, test, and produce). In metabolic engineering, the selected design cycle is constrained by the sheer number of bacterial host variants that require examination before the optimum variant is identified. The throughput of the design cycle directly influences target product development and its rate of production. Therefore, it is essential to identify where the bottlenecks are and overcome them. A detailed understanding of metabolic pathway dynamics helps a lot in the successful implementation of the aforesaid approaches. All the required pathway enzyme functionalities, omics relations with other components, gene regulatory agents, signal transduction and enzyme-protein interactions, and protein-protein interactions network knowledge facilitate researchers' ability to apply metabolic engineering based on more sophisticated and advanced technology. A constraint-based reconstruction and analysis tool provides metabolic engineering to recognize key enzymes and genes through genome-scale modelling of metabolic networks (King et al., 2015).

Furthermore, kinetic modeling and pathway design benefit from analysis and improvements in the thermodynamic feasibility of pathways reactions and fluxes. Flux balance

analysis and metabolic control analysis are widely used together to determine any reaction fluxes using mass balance and objective function equations. Implementing such *in silico* modeling is likely to aid in production maximization. Moreover, artificial intelligence and machine learning can be used to determine appropriate genes for manipulating pathway dynamics. This technique produced significantly better results from qualitative and quantitative predictions than conventional kinetic modeling (Choi et al., 2019; Costello and Martin, 2018). Gathering the required information using the proposed tools may be effective in boosting production via metabolic engineering.

As previously discussed, altering the natural metabolism of the glycosylation pathway in microorganisms is always challenging. The genetically engineered gene introduction via insertion and deletion and manipulation of gene expression for the production of the glycosyltransferase enzyme causes a notable harmful effect on the fitness of the host. Moreover, metabolic pathways compatible with one organism are not necessarily compatible with other host organisms and reduce the expression of essential native proteins. Therefore, to establish all such modifications, detailed knowledge of the necessary enzymes and proteins that participate in metabolic pathway reactions is required. The main challenge faced by researchers is the identification of suitable donor and host organisms due to a lack of information, as not many organisms have been sequenced and characterised at the genome, transcriptome, and metabolome levels, which makes it difficult for genetic modifications. The most frequently occurring problem related to selected host organisms is the production and accumulation of toxic intermediates due to modifications in metabolic pathways and flux. Such toxic intermediates exert feedback inhibition on the final product. Added to this, in large-scale microbial culture processes, the production of certain compounds imparts toxicity to the microorganism. However, removing these toxic compounds before reaching the toxic threshold level using the correct design of the bioreactor is usually a helpful method. This imposes further pressure on selecting those microorganism strains that can tolerate greater concentrations of these toxic compounds (Garcia-Granados et al., 2019). We need more and more projects, particularly for biological component characterization such as determining promoter strength, terminators, the average life of proteins and transcripts, and catalytic activity (Le Novere, 2015). Following this information, the creation of mathematical and computational models facilitates new researchers for better understanding of pathway system behaviour.

7. Conclusion

Metabolic glycan engineering strategies are advancing to the next level after identifying several types of N- and O-linked protein glycosylation machinery in various bacterial species and due to the development of techniques and tools that assist in the transfer of that functional system into genetically modified *E. coli* cells. Moreover, major advances in genomics, a vast pathway database, *in-silico* modelling, and mature nuclease-based gene editing analytical tools allow more precise alteration of target glycans and their metabolic fluxes. Many recent research studies have demonstrated great potential for tailored glycan production in *E. coli* by designing and constructing orthogonal pathways that significantly increased cell metabolism, relevant enzyme expression, yield, and productivity at scale. These efforts establish bacterial glycoprotein engineering platforms as promising strategies for generating more efficacious glycoconjugates and well-defined therapeutic compounds. Here we summarise the overall strategies of metabolic glycan engineering, some of the latest tools to identify metabolic bottlenecks in host cells, and target and non-relevant genes to manipulate the development of high-yielding bacterial cell factories. Additionally, various tailored glycans produced and their versatile applications were discussed, along with a proposed futuristic perspective on improving metabolic glycan engineering.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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Figures

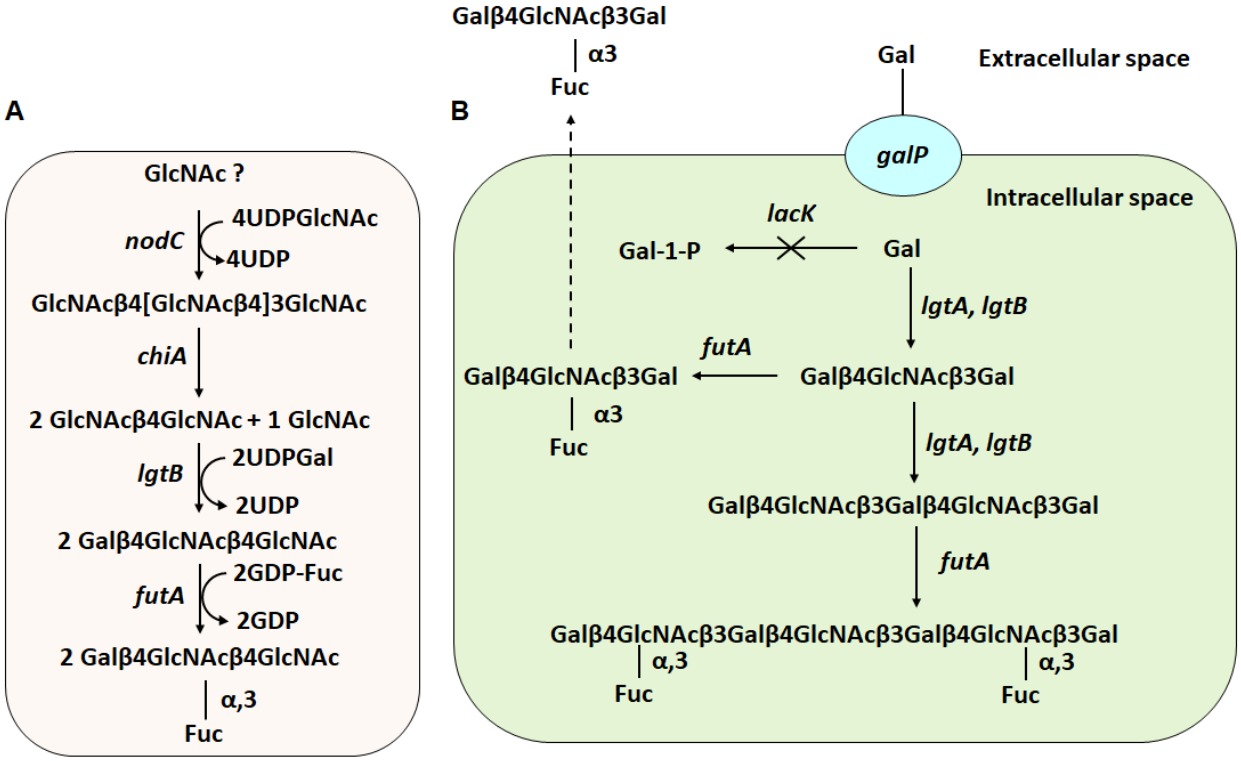


Figure 1. Elucidation of metabolic pathway engineering in *E. coli* for the production of Le^X-containing trisaccharides. (A) Chitobiose is produced in the cytoplasm of *E. coli* with the help of by chitin-synthase (nodC gene) and chitinase A1 gene (chiA). Then, the glycosylation of chitobiose by Galβ4T (lgtB gene) formes Galβ4GlcNAcβ4GlcNAc (Dumon et al., 2006). (B) Galβ4(Fucα3)GlcNAcβ3Gal is produced from exogenously supplied Gal by a mutant strain, deficient in galactokinase activity and upregulated expression of iGnT (lgtA gene) (Dumon et al., 2006). Please see the abbreviation list of Table 1 for full form of glycans.

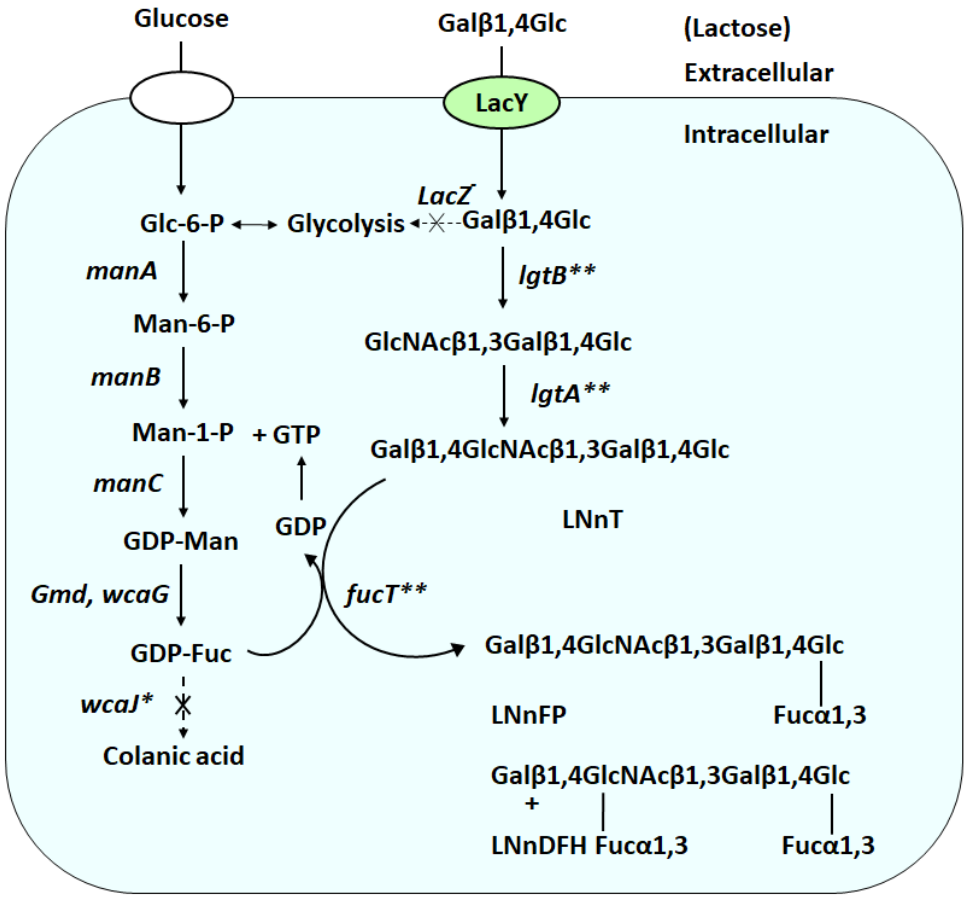


Figure 2. Metabolic pathway engineering in *E. coli* for the production of fucosylated-N-acetyllactosamine oligosaccharides. In order to produce fucosylated-N-acetyllactosamine oligosaccharides in vivo, *N. meningitidis* genes lgtAB and *H. pylori*'s α-1,3 fucosyltransferase (fucT) are heterologously expressed in *E. coli*. Downregulated genes are colanic acid synthesis wcaJ and lacZ. lgtA and lgtB are used to transfer GlcNAc and Gal, respectively. A potential acceptor for α-1,3 fucosyltransferase is lacto-N-neotetraose (LNnT), which can produce lacto-N-neo-fucopentaose (LNnFP) and lacto-N-neo-di fuco-hexaose (LNnDFH) (Dumon et al., 2001). *Inactivated endogenous genes; **heterologous genes carried on expression vectors. Please see the abbreviation list of Table 1 for full form of glycans.

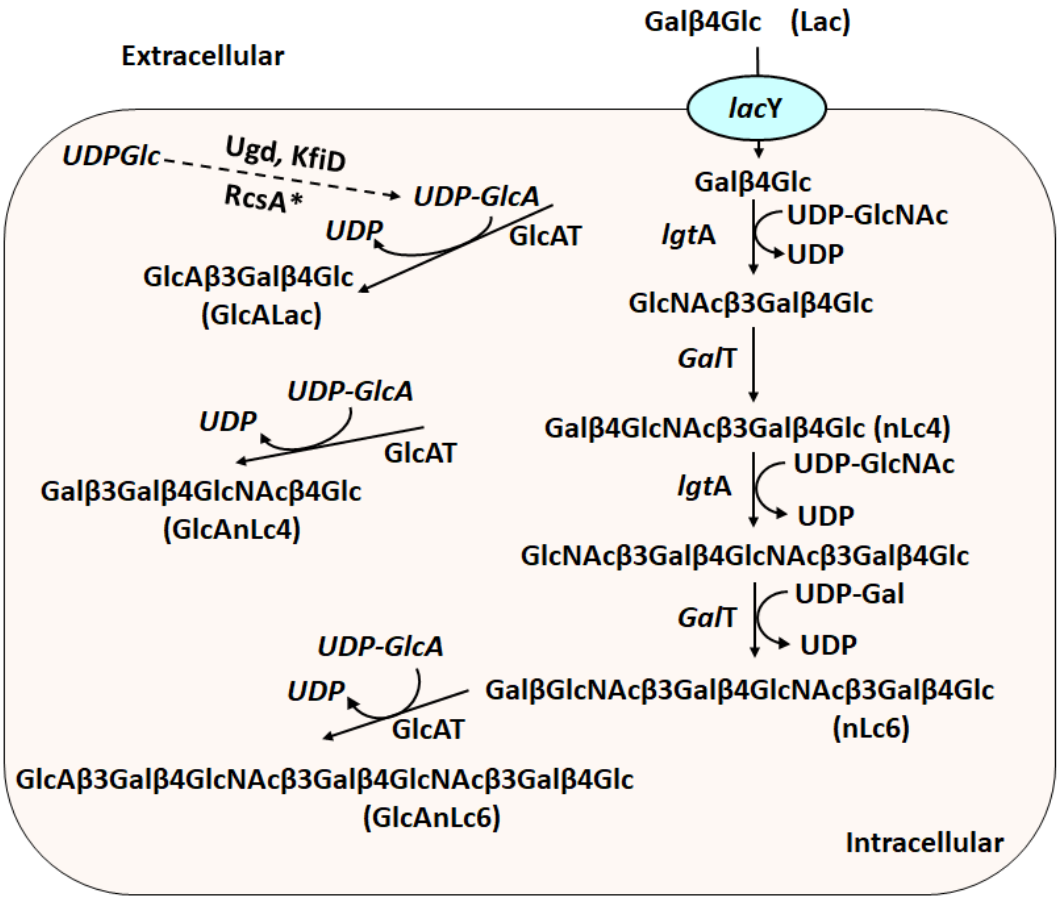


Figure 3. Metabolic pathway engineering in *E. coli* for the production of neolacto-oligosaccharides. Glucuronyl transferase (GlcATP) catalytic domain is expressed in an *E. coli* having lacY (β-galactoside) but it is lacking lacZ gene. GlcAnLc4 is glycosylated by the *H. pylori*'s β-1,3-GlcNAcT and *N. meningitides*'s β-1,4GalT. The glucuronyltransferase (GlcAT) uses endogenous Lac, nLc4 and nLc6 as substrates for the formation of GlcALac, GlcAnLc4 and GlcAnLc6, respectively (Yavuz et al., 2008). The synthesis of UDP-GlcA is catalysed by UDP glucose dehydrogenases of *E. coli* strains K5 (KfiD) and K-12 (Ugd). *Cofactor act as positive regulator of Ugd gene.

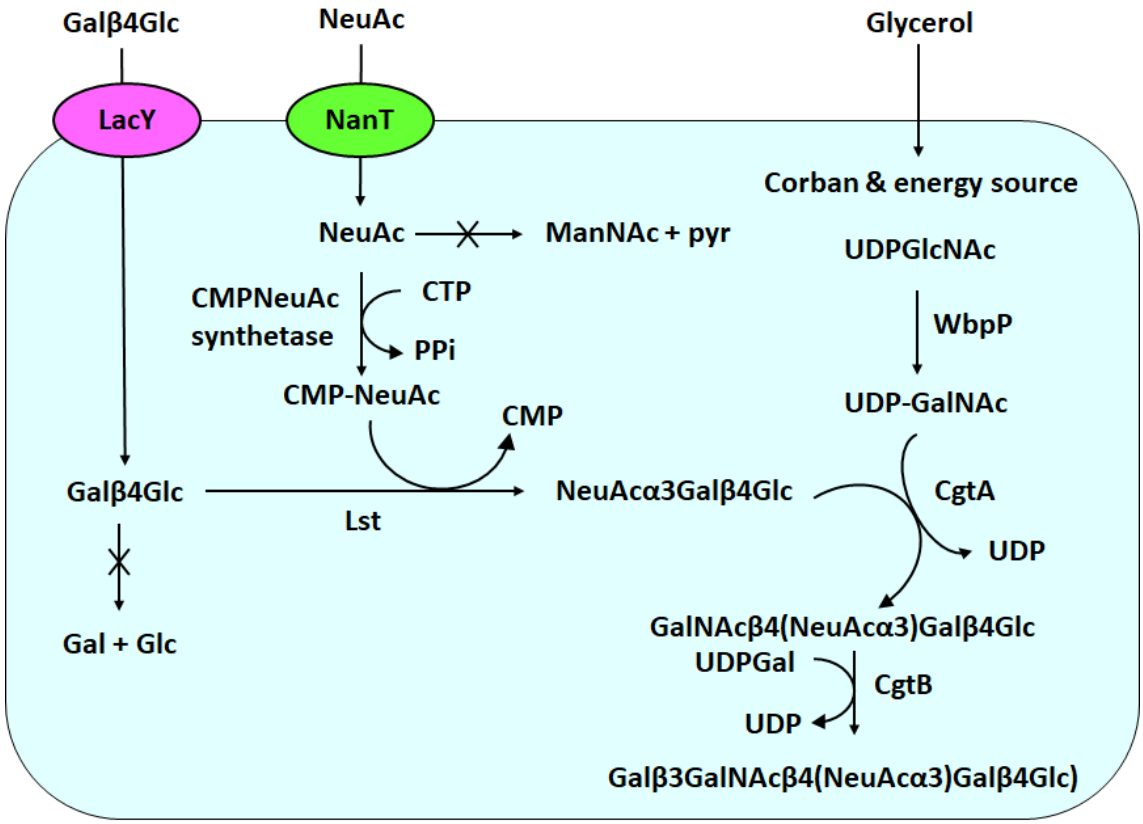

Galβ4Glc
NeuAc
Glycerol
LacY
NanT
Corban & energy source
NeuAc
ManNAc + pyr
UDPGlcNAc
CMPNeuAc
synthetase
CTP
PPi
WbpP
CMP-NeuAc
UDP-GalNAc
CMP
Galβ4Glc
NeuAcα3Galβ4Glc
CgtA
Lst
UDP
Gal + Glc
GalNAcβ4(NeuAcα3)Galβ4Glc
UDPGal
CgtB
UDP
Galβ3GalNAcβ4(NeuAcα3)Galβ4Glc)

Figure 4. Metabolic pathway engineering in *E. coli* for the production of different oligosaccharides. The permeases LacY and NanT, which transport lactose and NeuAc inside the cell, shield it from further degradation brought on by the inactivation of the β-galactosidase (LacZ) and aldolase (NanA) genes. CMP-NeuAc synthase converts NeuAc into CMPNeuAc, which is subsequently transferred onto lactose by 2,3-sialyltransferase (Lst), resulting in the formation of sialyllactose. β1,4-GalNAc transferase (CgtA) utilizes the recombinant UDPGlcNAc C4 epimerase (WbpP) producing endogenous pool of UDPGalNAc to catalyse the glycosylation of sialyllactose to form *II*^{3}*Neu5Ac-GgOse*$_{3}$. This substance acts as an acceptor for β1,3 galactosyltransferase (CgtB) to produce *II*^{3}*Neu5Ac-GgOse*$_{4}$ (Antoine et al., 2005). CTP, cytidine triphosphate; PPi, inorganic triphosphate.

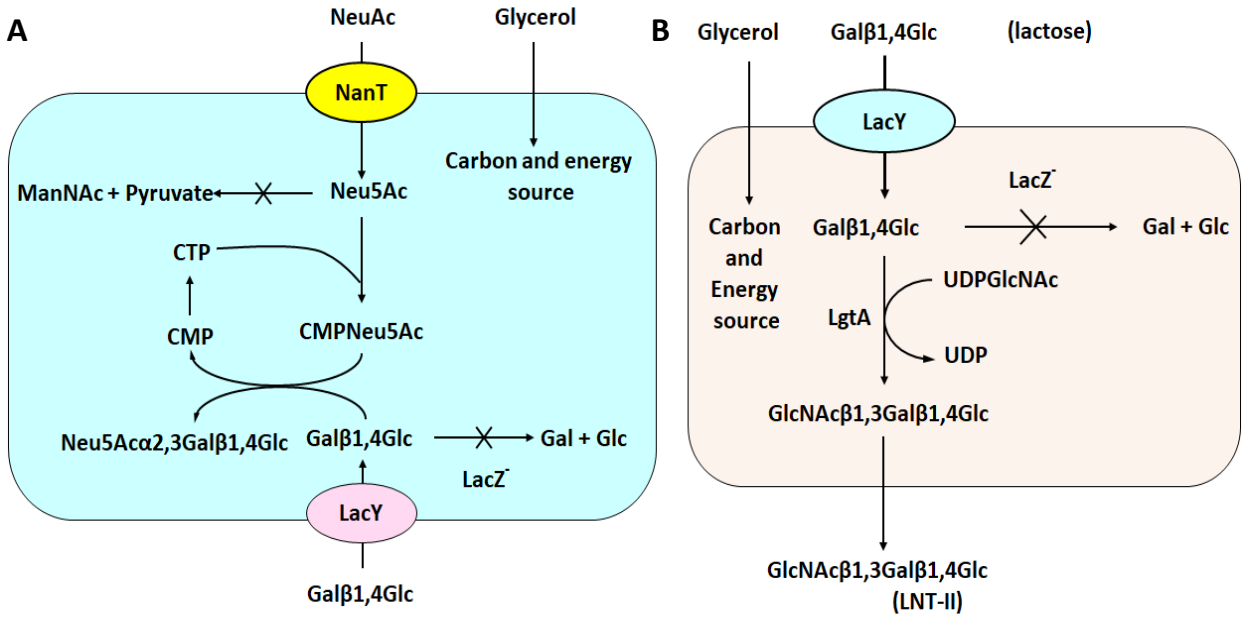


Figure 5. Representation of metabolic pathway engineering in *E. coli* for the production of oligosaccharides. (A) CMPNeu5Ac synthase and the α2,3 sialyltransferase genes expressing *E. coli* strain synthesizes 3′ sialyllactose (Neu5NAcα2-3Galβ1-4Glc). The production of CMP-

Neu5Ac synthase and β-2,3 sialyltransferase enables the intracellular activation of NeuAc into CMP-Neu5Ac and its ensuing transfer onto lactose. (B) *E. coli* expressing the lgtA gene synthesizes trisaccharide LNT-II (GlcNAcβ1-3Galβ1-4Glc) and prevents lactose degradation due to the cell's lack of β-galactosidase activity (Priem et al., 2002).

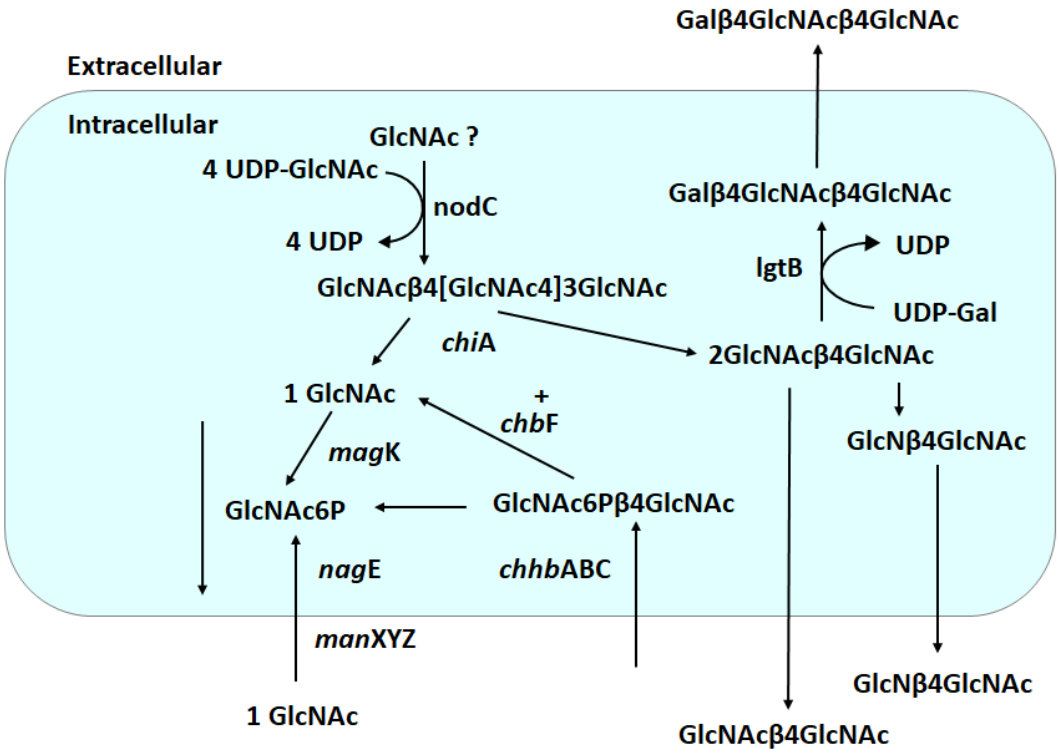


Figure 6. Metabolic pathway engineering in *E. coli* for the production of chitobiose. Co-expression of the chitinase gene chiA and the rhizobial chitinoligosaccharide synthase gene nodC synthesize N^{I},N^{II}-diacetylchitobiose (chitobiose). The PTS permease ChbBCA phosphorylated chitobiose to recover it primarily. The expression of β1,4-galactosyltransferase (lgtB) converts intracellular chitobiose into the trisaccharide Galβ4GlcNAcb4GlcNAc. This compound might behave as an acceptor glycosyltransferases that identify the terminal N-acetyllactosamine structure (Cottaz and Samain, 2005).

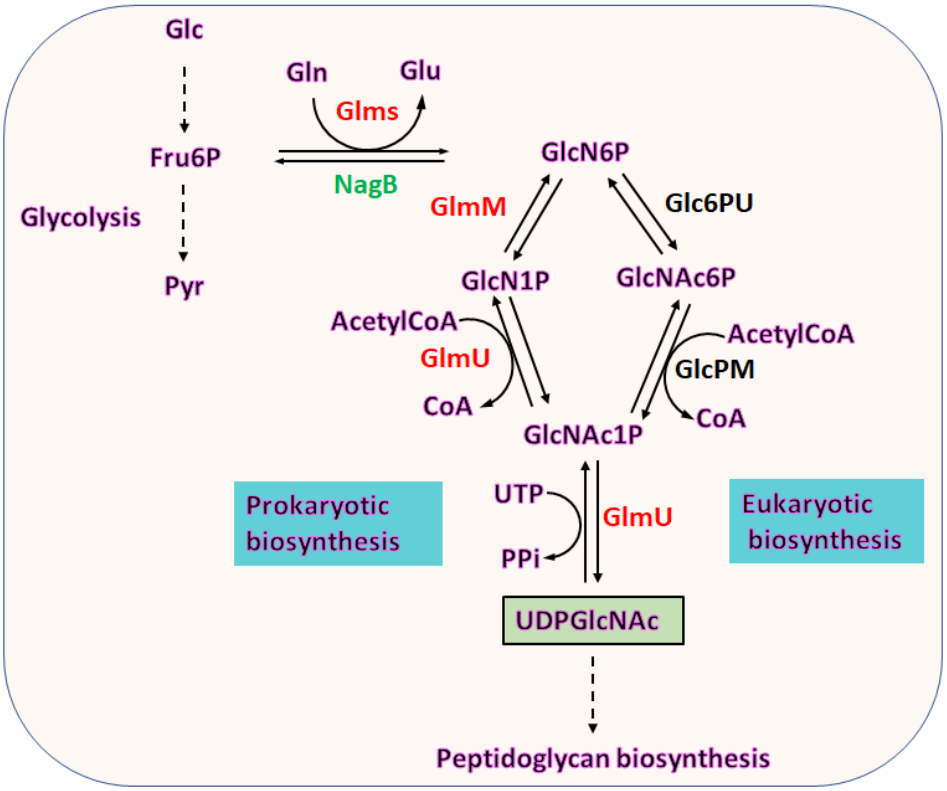


Figure 7. An illustration of the metabolic pathway used by *C. glutamicum* to produce UDP-GlcNAc and the metabolic engineering techniques used to manufacture excessive UDP-GlcNAc (Gauttam et al., 2021). Red or green text is used to indicate the outcomes of overproducing or knocked-out genes, respectively.

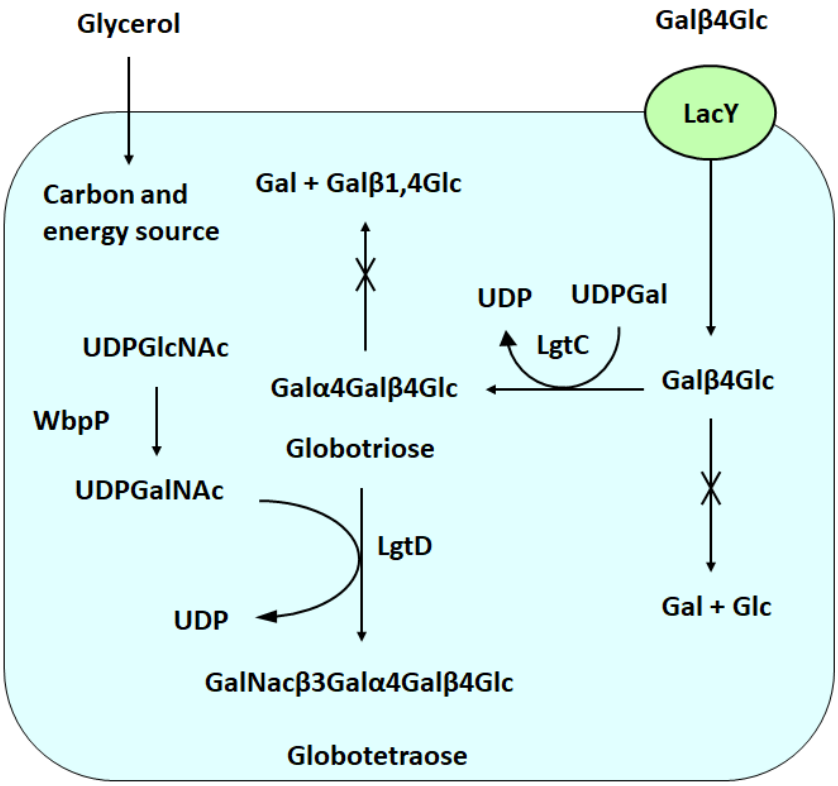


Figure 8. Metabolic pathway engineering in *E. coli* for the production of globotriose and globotetraose. The transfer of α1,4Gal transferase to *E. coli* by lgtC gene from *N. meningitides.* Two additional genes, WbpP of *P. aeruginosa* (which encodes UDPGalNAc C4 epimerase) and IgtD from *Hemophilus influenzae* RD (which β1,3GalNAc transferase), are overexpressed in *E. coli* to synthesize globotetraose. The melA gene (which encodes α-galactosidase) is excluded to prevent globotriose degradation. The β3GalNAc transferase (LgtD) converts globotriose to globotetraose (Antoine et al., 2005).

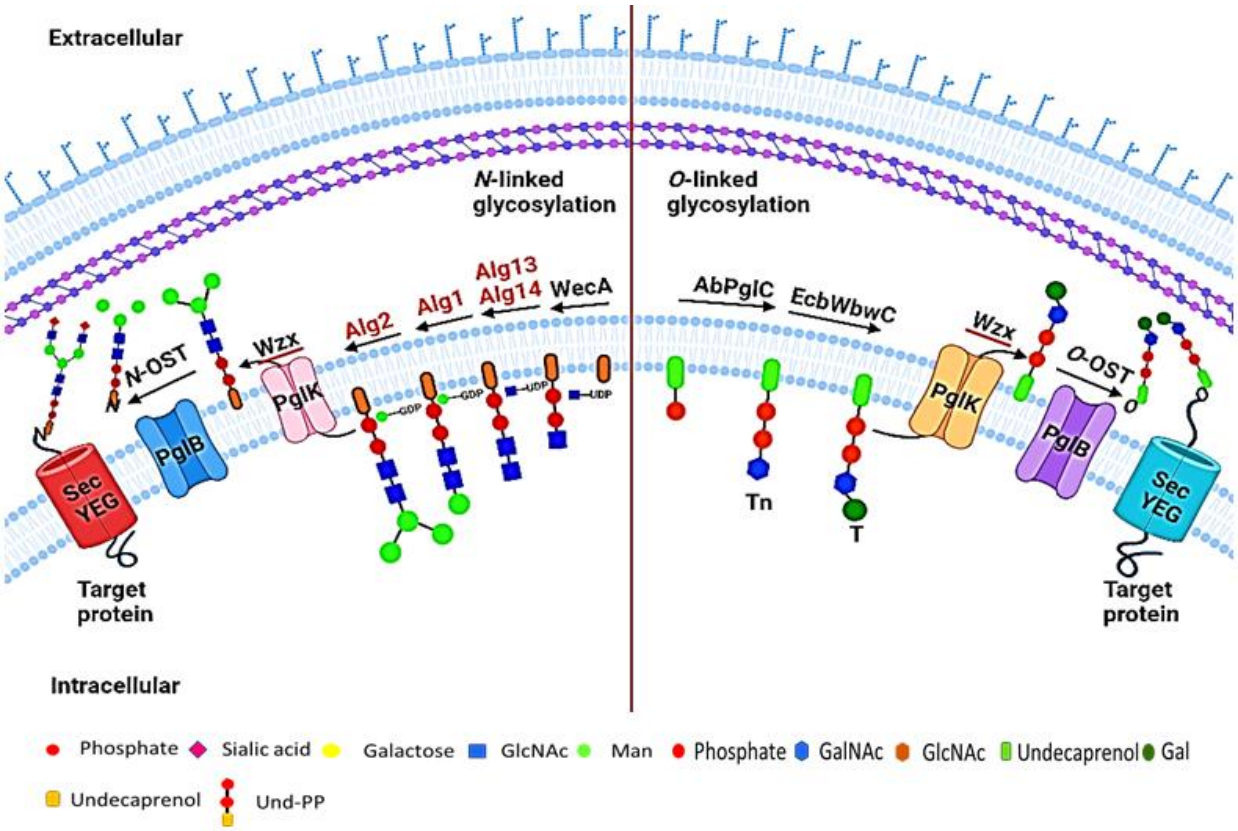
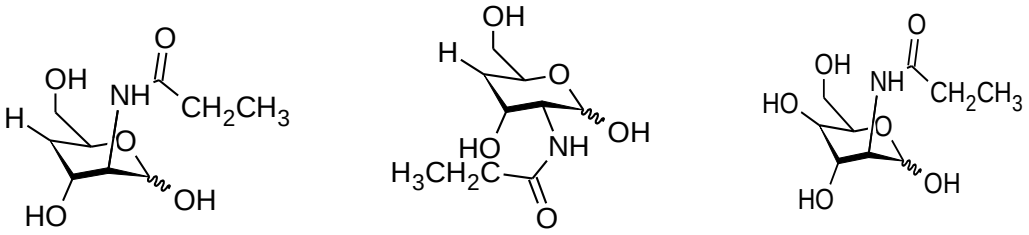
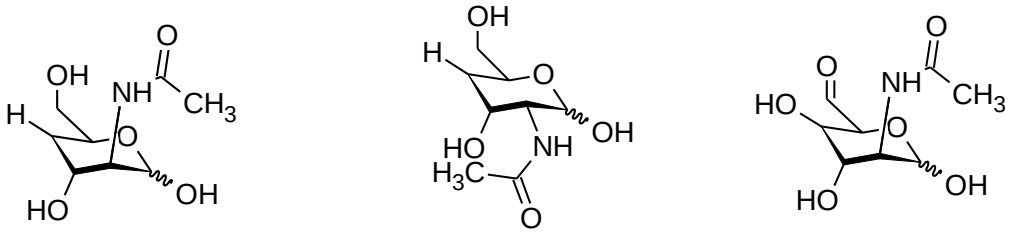


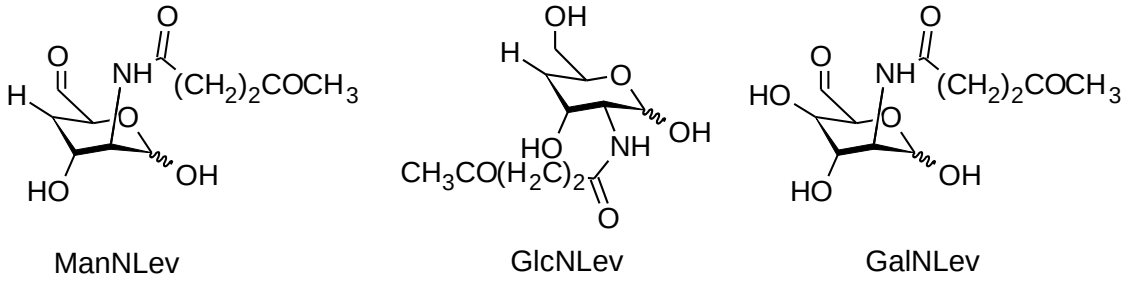
Figure 9. Representation of *O*- and *N*-linked glycosylation pathways in *E. coli*. *O*-linked glycosylation initiated with the formation of the Und-PP-linked GalNAc by UDP-GalNAc-dependent *AbP*glC. *Ec*WbwC extends Und-PP-GalNAc by a single Gal residue, forming lipid-linked Galβ-1,3-GalNAc formation. To transfer the preassembled T antigen glycan all at once to a serine amino acid on a Sec pathway-exported acceptor protein, the *E. coli* flippase Wzx flips the lipid-linked oligosaccharide to the cytoplasmic membrane (Natarajan et al., 2020). For *N*-linked glycosylation, an endogenous glycosyltransferase WeCA converts GlcNAc1 phosphate to undecaprenyl phosphate. Alg13 and Alg14 subunits of *S. cerevisiae*'s β1,4GlcNAc transferase were used to add the second GlcNAc residue to GlcNAc-PP-Und. The first mannose is added to the glycan by the β1,4-mannosyltransferase (Alg1), and the bifunctional mannosyltransferase (Alg2) adds a α1,3-mannose and a α1,6-mannose in a branching configuration. Flippase (PlgK) transfers lipid and sugar residues towards periplasmic phase. The integral membrane protein PlgB, a single subunit, exhibits *N*-OST activity to transfer the sugar moiety to the protein residue either co-translationally or post-translationally (Valderrama-Rincon et al. 2012).



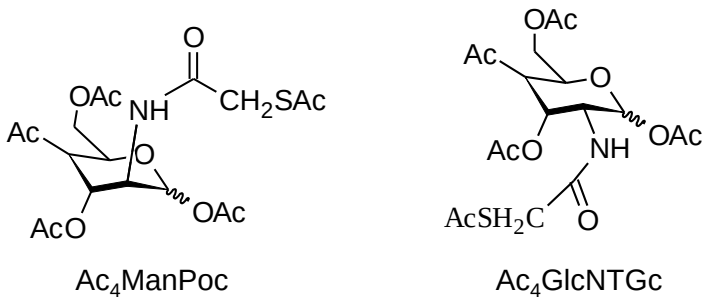
Aliphatic analogues



Natural glycans



Ketones



Thiols

Figure 10. Aliphatic analogues, natural glycans, ketones and thiols as chemical probes for glycoengineering. Please see the abbreviation list of Table 1 for full form of glycans.

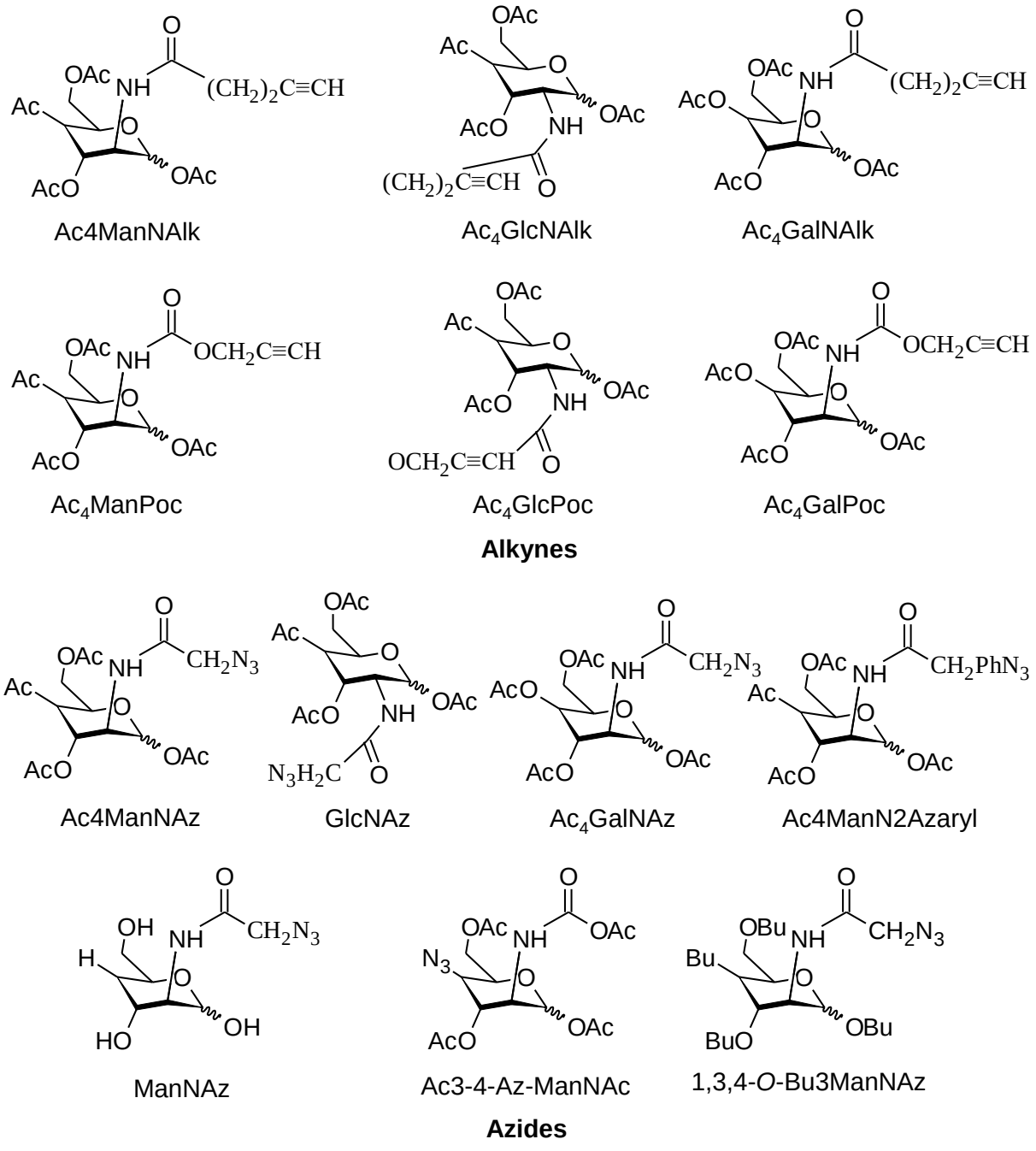


Figure 11. Alkynes and azides as chemical probes for glycoengineering. Please see the abbreviation list of Table 1 for full form of glycans.

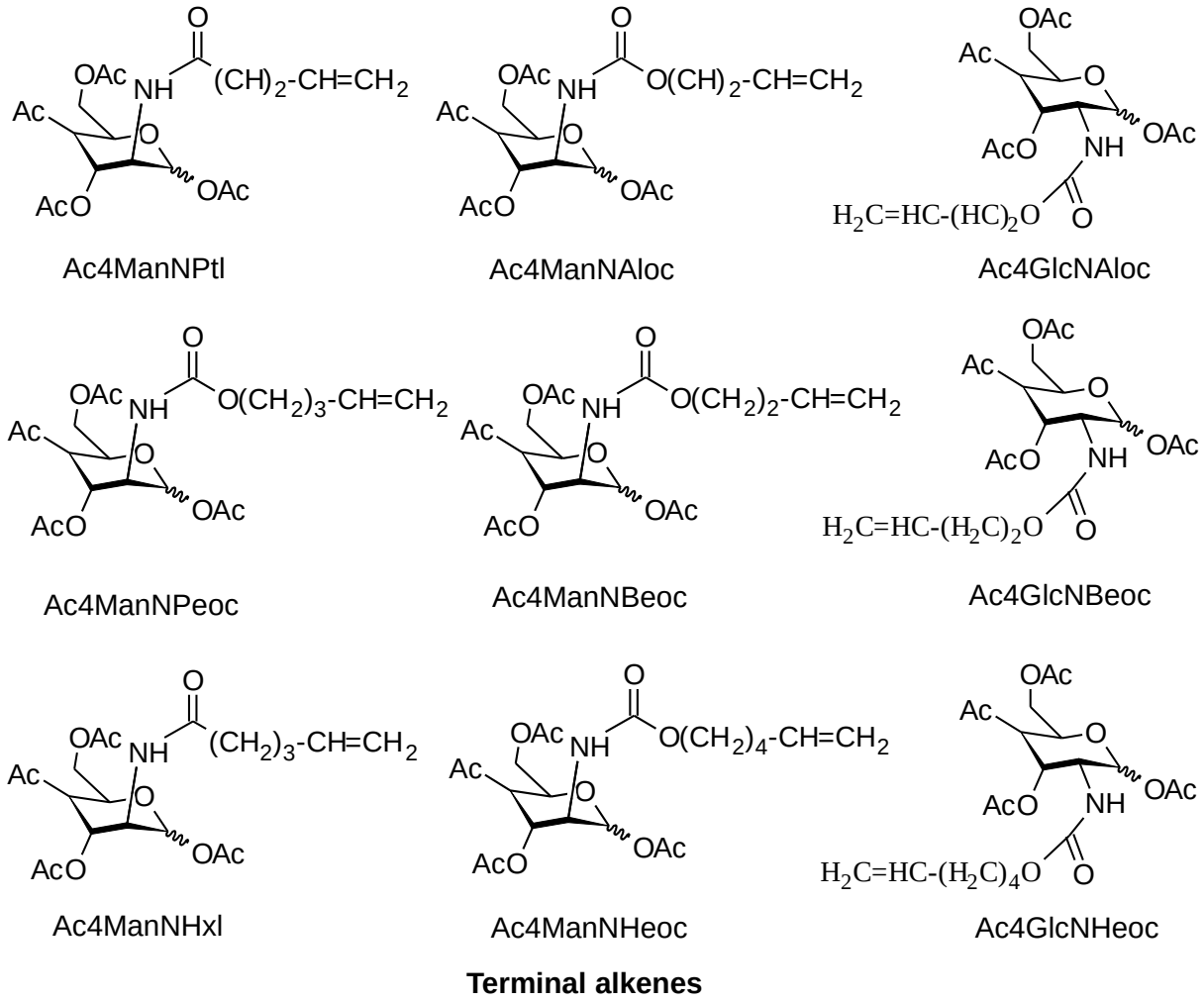


Figure 12. Terminal alkynes as chemical probes for glycoengineering. Please see the abbreviation list of Table 1 for full form of glycans.

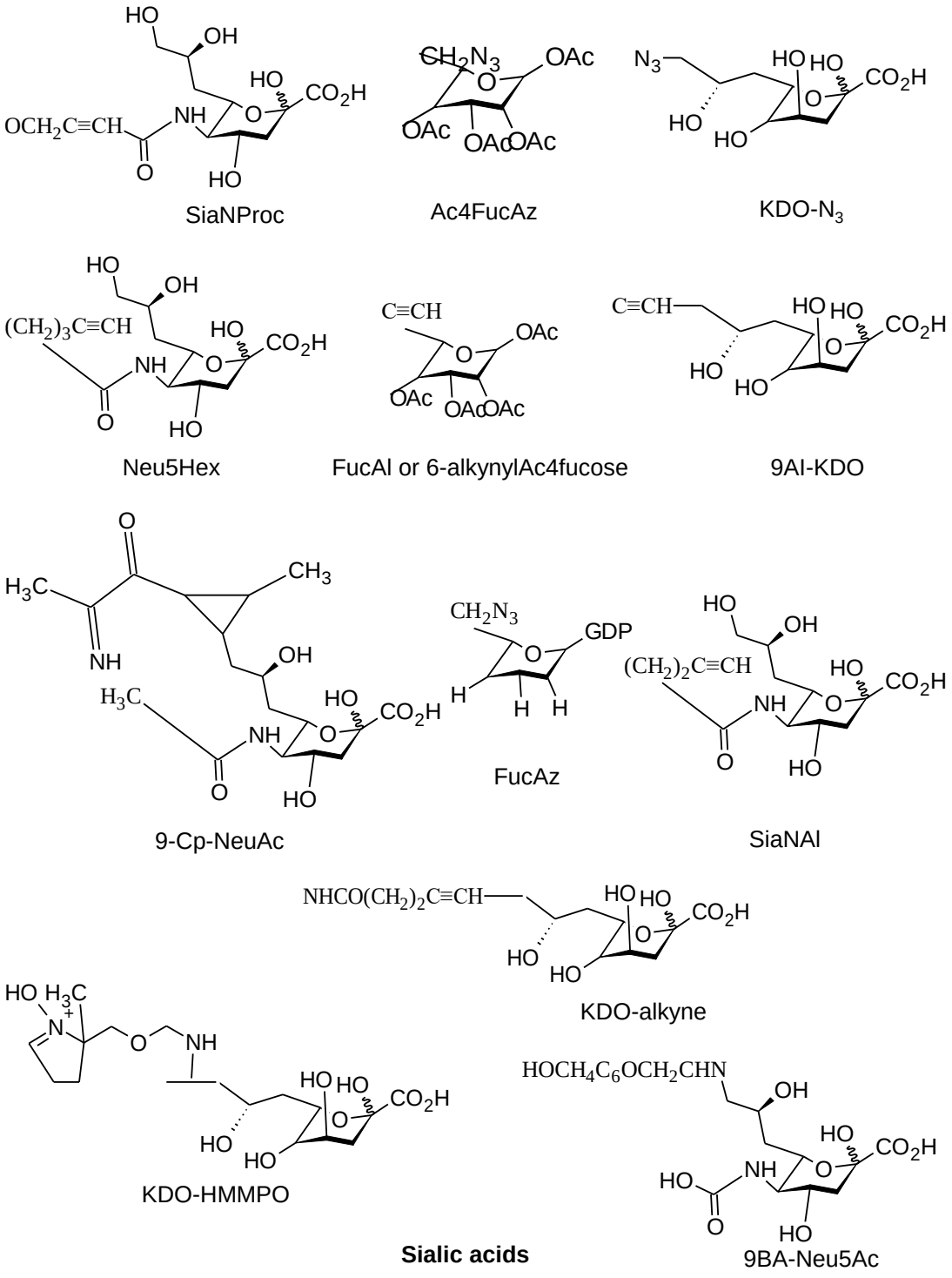
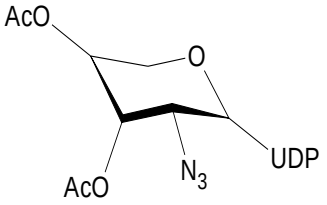
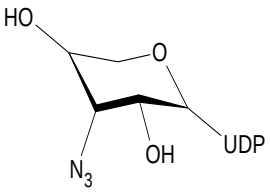


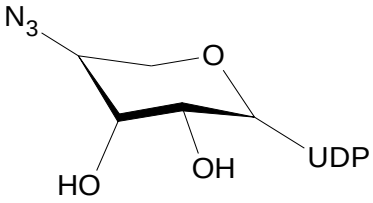
Figure 13. Sialic acids as chemical probes for glycoengineering. Please see the abbreviation list of Table 1 for full form of glycans.



UDP-2-XylAz

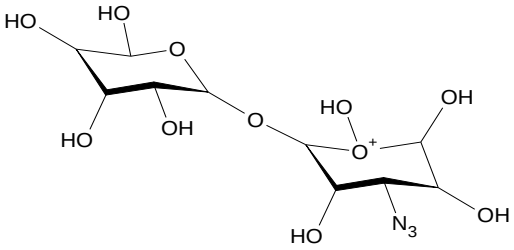


UDP-3-XylAz

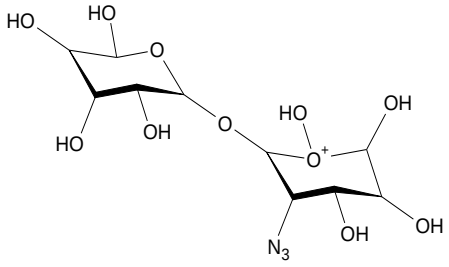


UDP-3-XylAz

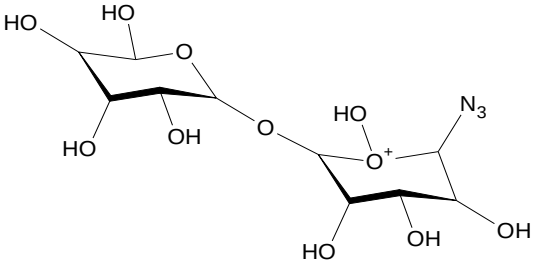
Xylose



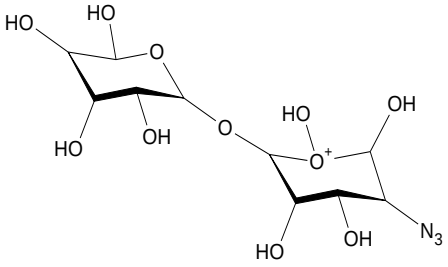
3-TreAz



2-TreAz

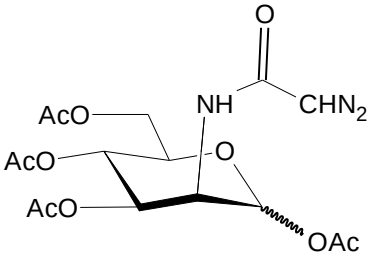


6-TreAz

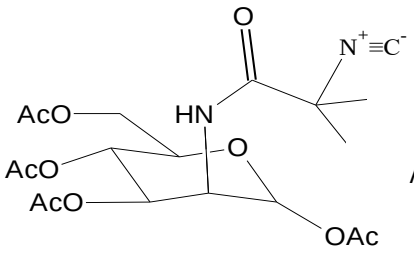


4-TreAz

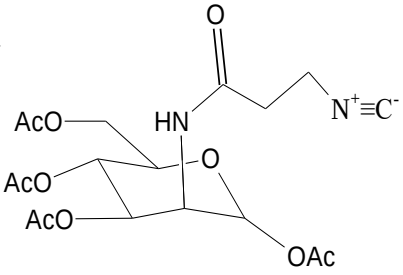
Trehalose



Ac_4GalDiaz



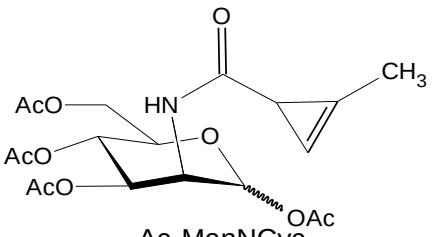
Ac4ManN-*t*-Iso



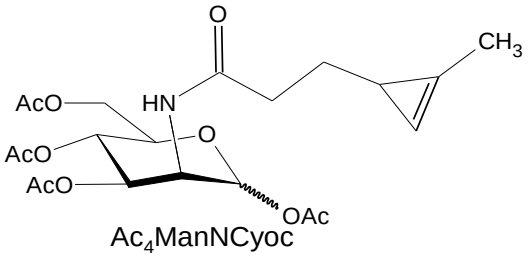
Ac4ManN-*n*-Iso

Diazoketones

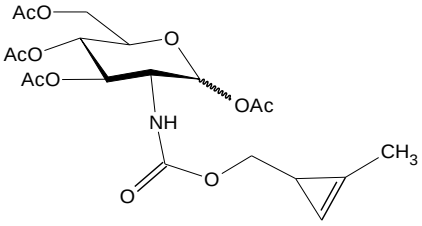
Figure 14. Xylose, trehalose and diazoketones as chemical probes for glycoengineering. Please see the abbreviation list of Table 1 for full form of glycans.



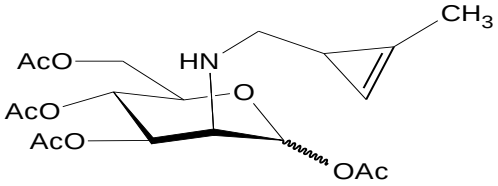
$Ac_4ManNCyc$



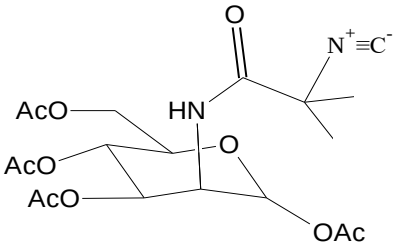
$Ac_4ManNCyoc$



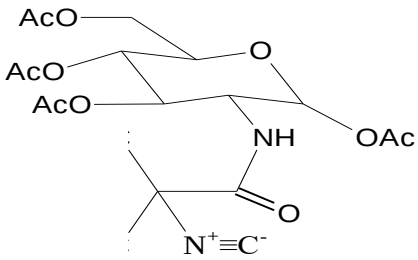
$Ac_4GlcNCyoc$



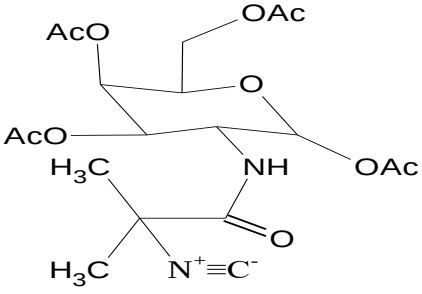
$Ac_4GalNCyoc$



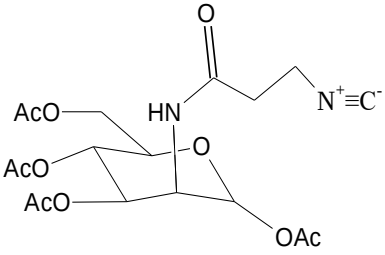
Ac_4ManN-*t*-Iso



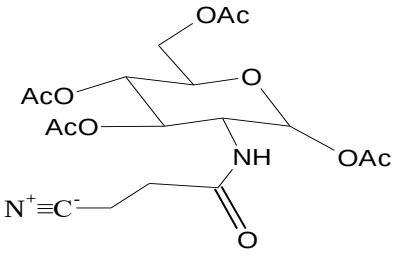
Ac_4GlcN-*t*-Iso



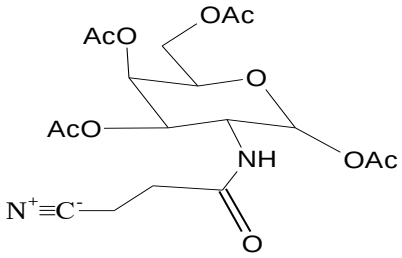
Ac_4GalN-*t*-Iso



Ac_4ManN-*n*-Iso



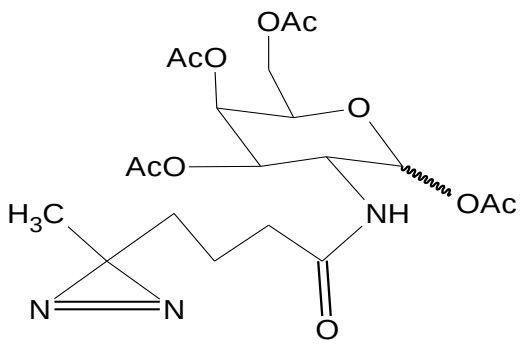
Ac_4GlcN-*n*-Iso



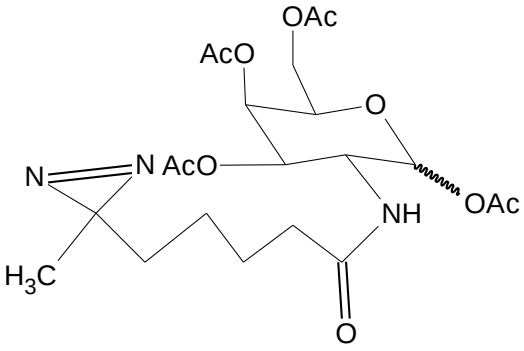
Ac_4GalN-*n*-Iso

Cyclopropenes

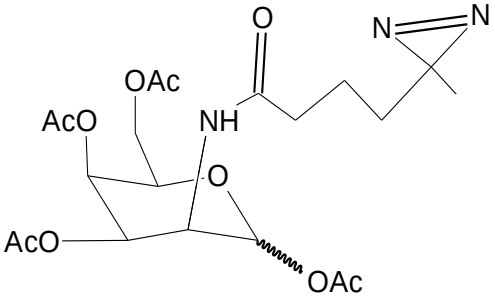
Figure 15. Cyclopropenes as chemical probes for glycoengineering. Please see the abbreviation list of Table 1 for full form of glycans



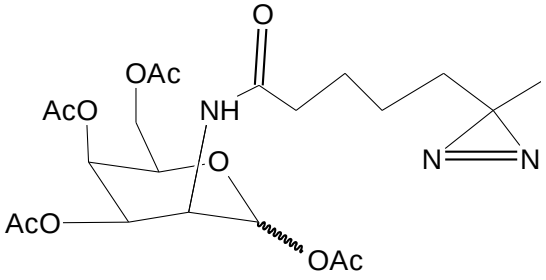
$Ac_4GlcNDAz(3me)$



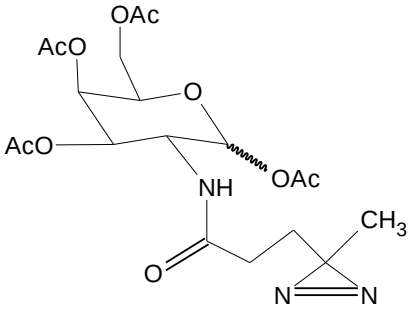
$Ac_4GlcNDAz(4me)$



$Ac_4ManNDAz$- (3me)

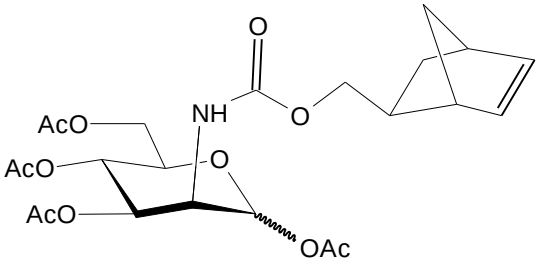


$Ac_4ManNDAz(4me)$

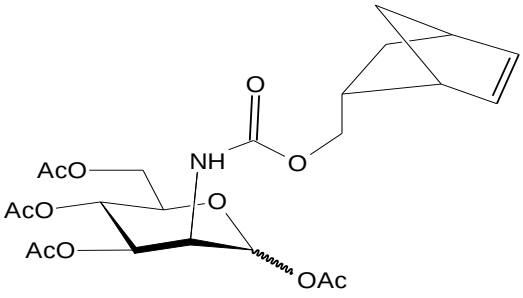


$Ac_4GalNDAz(2me)$

Diazirines



$Ac_4ManNNorbocendo$



$Ac_4ManNNorbocexo$

Norbornenes

Figure 16. Diazirines as chemical probes for glycoengineering. Please see the abbreviation list of Table 1 for full form of glycans

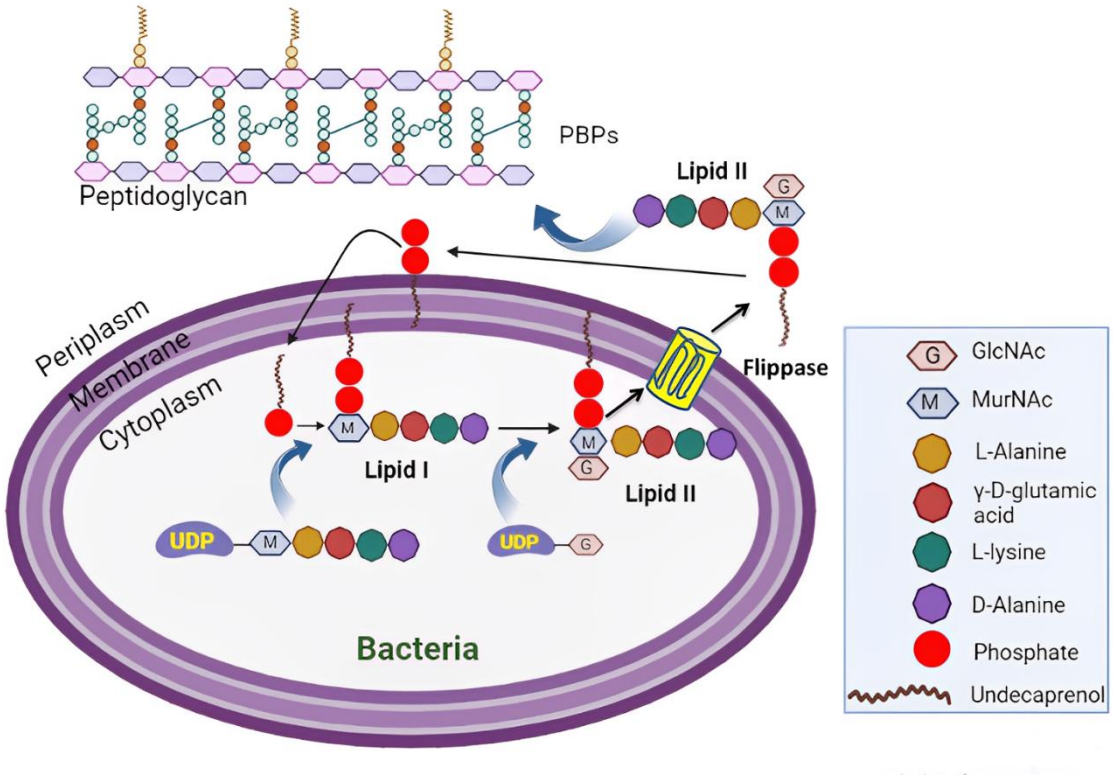


Figure 17. Peptidoglycan (PG) biosynthetic pathway, showing nucleotide activated UDP-GlcNAc transferred onto Lipid I to form Lipid II. Where, Lipid II act as precursor for peptidoglycan synthases, catalyzed PG enlargement during cell growth and cell division (Xu et al., 2022).

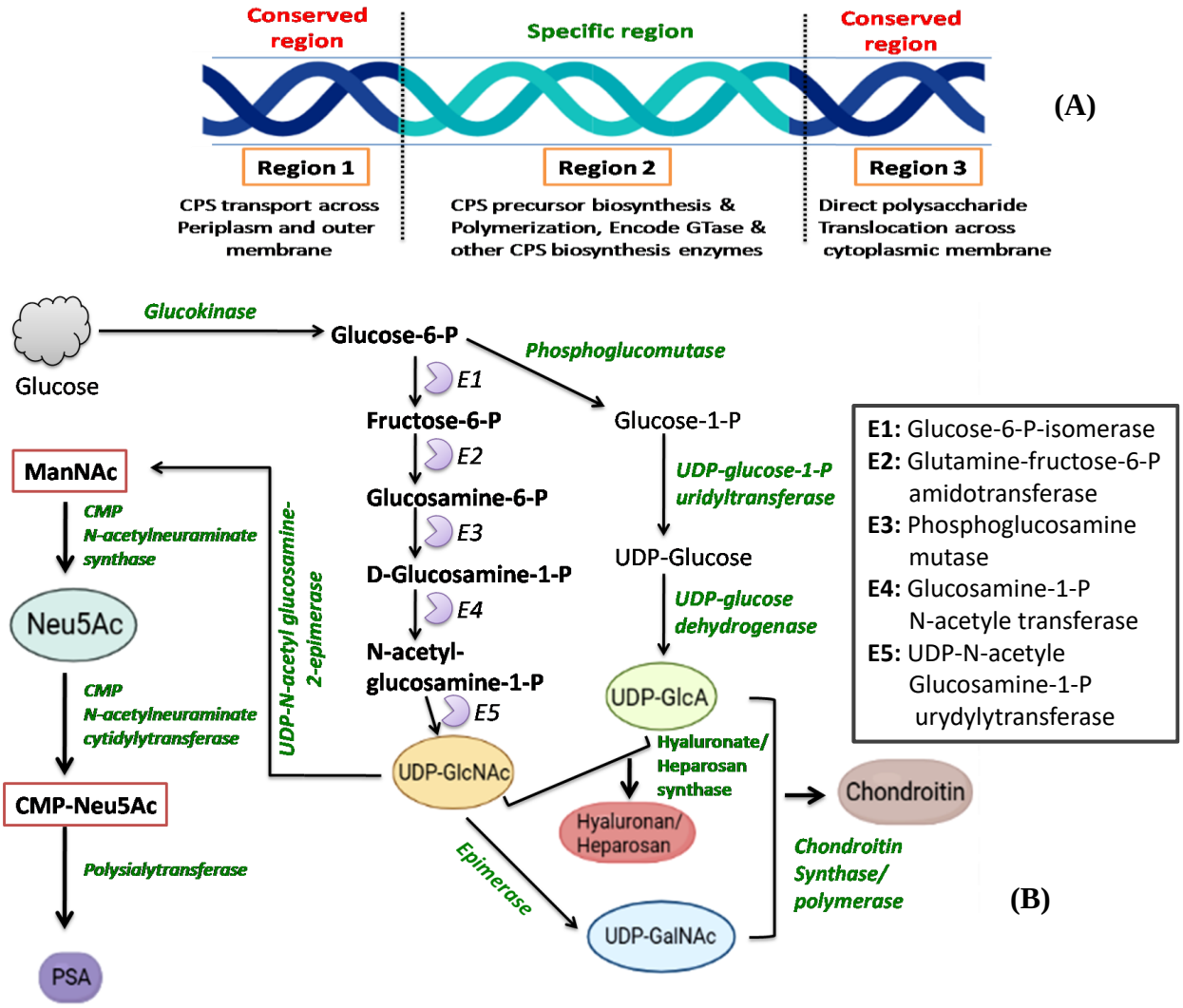


Figure 18. Representation of biosynthetic pathways for capsular polysaccharide (CPS) production in *E. coli*. (A) Location of genes at different region of conserved and specific regions. (B) Schematic representation of CPSs biosynthesis pathways in bacteria. PSA hyaluronan/heparosan and chondroitin are CPSs product. Neu5Ac, UDP-GlcNAc, UDP-GalNAc and UDP-GlcA are non-sulfated GAG precursor subunits (Williams et al., 2018). Enzymes responsible for catalyzing each reaction are represented by green color (Italic naming).

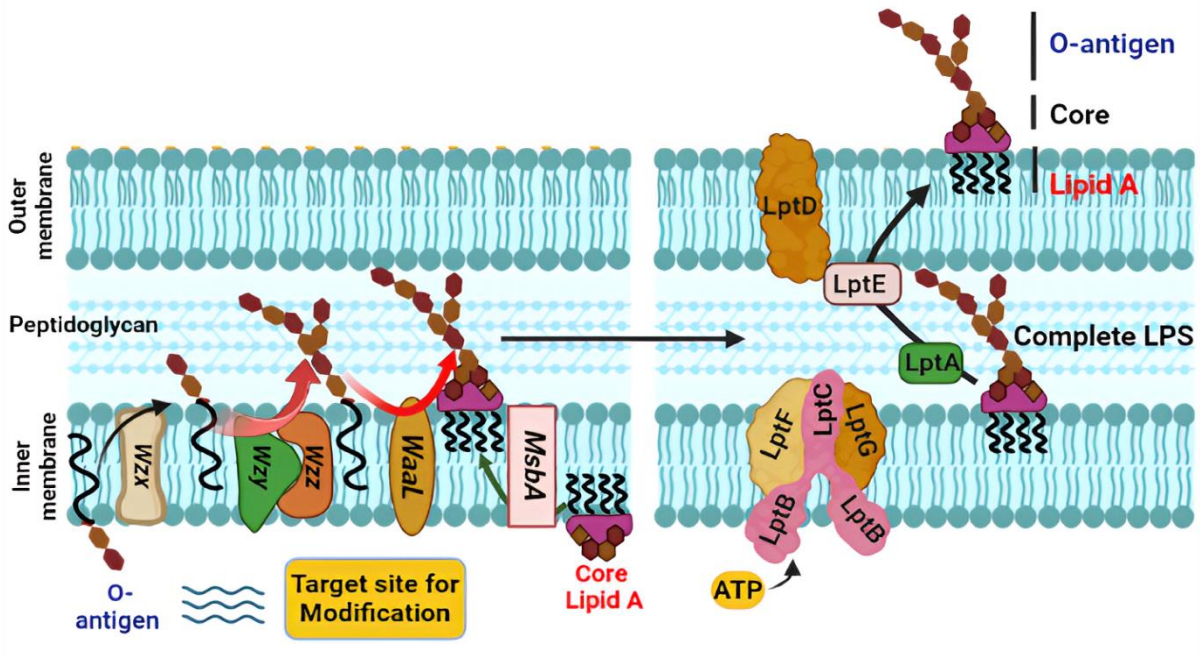
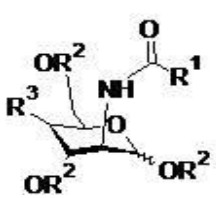
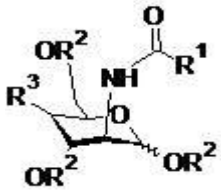
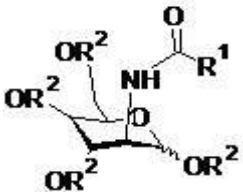
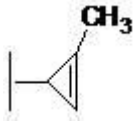
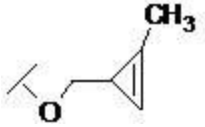
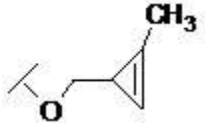
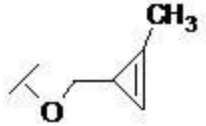
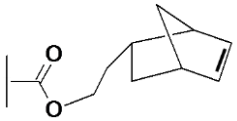
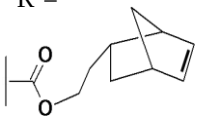


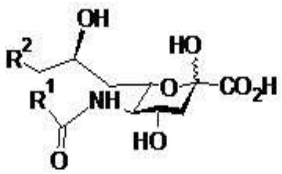
Figure 19. LPS biosynthetic pathway for metabolic glycans engineering in *E. coli*. Wzy polymerizes the chain up to the length specified by Wzz, and WaaL aids in the ligation of the chain onto complete core-Lipid A molecules, which are separately translocated by MsbA. This is followed by Wzx translocating O-antigen subunits across the inner membrane. The proteins LptA, B, C, D, E, F, and G then construct LPS molecules and transport them through the peptidoglycan and outer membrane. The outer core of LPS is often constituted of the most prevalent monosaccharides (glucose & galactose), while the inner core typically consists of two or three Kdo units (Ruiz et al., 2009).

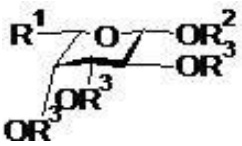
Table 1. List of glycan-based chemical probes for glycoengineering

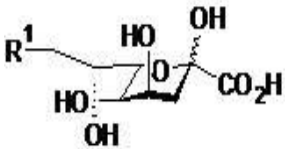
Chemical	Sugar analogs and molecular structure						Ref.
	Mannosamine 		**Glucosamine** 		**Galactosamine** 		
Class	Probe	Molecular structure	Probe	Molecular structure	Probe	Molecular structure	
Aliphatic analogues	ManNPro	R^1= CH^2CH_3 R^2= OR^3= H	GlcNPro	R^1= CH2CH3 R^2= H	GalNPro	R^1= CH2CH3 R^2= H	(Kayser et al., 1992)
Natural glycans	ManNAc	R^2= OR^3= H R^1= CH^3	GlcNAc	R^1= CH3 R^2= H	GalNAc	R^1= CH3 R^2= H	
Ketones	ManNLev	R^2= OR^3= H R^1= $(CH_2)_2COCH_3$	GlcNLev	R^2= H R^1= $(CH_2)_2COCH_3$	GalNLev	R^1= $(CH_2)_2COCH3$ R^2= H	(Mahal et al., 1997)
Alkynes	Ac_4ManNAlk	R^1= $(CH_2)_2C{\equiv}CH$ R2= OR3= Ac	Ac_4GlcNAlk	R^1= $(CH_2)_2C{\equiv}CH$ R^2= Ac	Ac_4GalNAlk	R^1= $(CH_2)_2C{\equiv}CH$ R^2= Ac	(Bateman et al., 2013;
	Ac_4ManPoc	R^2=OR^3=Ac R^1= $OCH_2C{\equiv}CH$	Ac_4GlcPoc	R^1= OCH2C≡CH R^2= Ac	Ac_4GalPoc	R^1= $OCH_2C{\equiv}CH$ R^2= Ac	Zaro et al., 2014)
Azides	Ac4ManNAz	R^1= CH_2N_3 R^2= OR^3= Ac	GlcNAz	R^1= CH_2N_3 R^2= H	Ac_4GalNAz	R^1= CH_2N_3 R^2= H	(Luchansky et al., 2004;
	Ac4ManN2Azaryl	R^1= CH_2PhN_3 R^2= OR^3= Ac					Saxon and Bertozzi,
	ManNAz	R^2= OR^3= H R^1= CH_2N_3					2000; Tian et al., 2015)
	Ac3-4-Az-ManNAc	R^1= OAc R^2= Ac R^3= N_3					
	1,3,4-*O*-Bu3ManNAz	R^2= R^3= Bu R^1= CH_2N_3					
Thiols	Ac_4ManPoc	R_1=CH_2SAc	Ac_4GlcNTGc	R^1= CH_2SAc			(Moller et al., 2012)

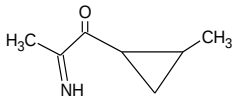
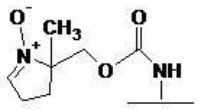
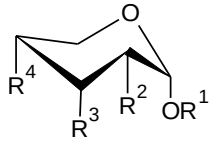
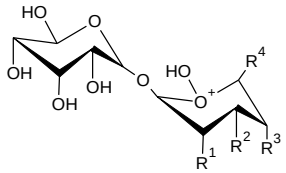
		R_2=OR_3=Ac		R^2= Ac			
Terminal alkenes	Ac4ManNPtl	R^1= $(CH_2)_2$ – $CH=CH_2$ R^2= OR^3= Ac					(Beckmann et al., 2012; Niederwieser et al., 2013; Spate et al., 2014)
	Ac4ManNAloc	R^1= OCH^2– $CH=CH^2$ R^2= OR^3= Ac	Ac4GlcNAloc	R^1= OCH_2–$CH=CH_2$ R^2= Ac			
	Ac4ManNPeoc	R^1= $O(CH_2)_3$– $CH=CH_2$ R^2= OR^3= Ac	Ac4GlcNPeoc	R^1= $O(CH_2)_3$– $CH=CH_2$ R^2= Ac			
	Ac4ManNHxl	R^1= $(CH_2)_3$ – $CH=CH_2$ R^2= OR^3= Ac					
	Ac4ManNBeoc	R^1= $O(CH_2)_2$– $CH=CH_2$ R^2= OR^3= Ac	Ac4GlcNBeoc	R^1= $O(CH_2)_2$– $CH=CH_2$ R^2= Ac			
	Ac4ManNHeoc	R^1= $O(CH_2)_4$– $CH=CH_2$ R^2= OR^3= Ac	Ac4GlcNHeoc	R^1= $O(CH_2)_4$– $CH=CH_2$ R^2= Ac			
Cyclopropene	Ac_4ManNCyc	 R^2= OR^3=Ac					
	Ac4ManNCyoc	R^1= 	Ac_4GlcNCyoc	R^1= 	Ac_4GalNCyoc	R^1= 	(Patterson et al., 2014)

		R^2= OR^3= Ac		R^2= Ac		R^2= Ac	
Isonitrile	Ac4ManN-*t*-Iso	R^1= $C(CH_3)_2CH_2$–N≡C R_2-OR_3=Ac	Ac_4GlcN-*t*-Iso	R^1= $C(CH_3)_2CH_2$–N≡C R^2= Ac	Ac_4GalN-*t*-Iso	R^1= $C(CH_3)_2CH_2$–N≡C R^2= Ac	(Stairs et al., 2013)
	Ac4ManN-*n*-Iso	R^1= $(CH_2)_2$ –N≡C R^2= OR^3= Ac	Ac_4GlcN-*n*-Iso	R^1= $(CH_2)_2$ –N≡C R^2= Ac	Ac_4GalN-*n*-Iso	R^1= $(CH_2)_2$ –N≡C R^2= Ac	
Diazirines	Ac4ManNDAz(4Me)	R^1= $(CH_2)_4$-1-methyldiazirinyl R^2= OR^3= Ac	Ac_4GlcNDAz(4Me)	R^1= $(CH_2)_4$-1-methyldiazirinyl R^2= Ac			(Ahad et al., 2013; Bond et al., 2011)
	Ac4ManNDAz(3Me)	R^1= $(CH_2)_3$-1-methyldiazirinyl R^2= OR^3= Ac	Ac_4GlcNDAz(3Me)	R^1= $(CH_2)_3$-1-methyldiazirinyl R^2= Ac	Ac_4GalNDAz(2Me)	R^1= $(CH_2)_2$-1-methyldiazirinyl R^2= Ac	
Norbornene	Ac4ManNNorbocendo	R^1= 					
	Ac4ManNNorbocexo	R^2=Ac R^1= 					
		R^2=Ac					
Diazoketones	Ac4ManN-t-Iso	R^1= $C(CH_3)_2CH_2$–N≡C R^2=OR^3=Ac			Ac_4GalDiaz	R^1= CHN_2 R^2= Ac	(Andersen et al., 2015; Josa-Cullere et al., 2014)
	Ac4ManN-*n*-Iso	R^1= $(CH_2)_2$ –N≡C R^2= OR^3= Ac					

Sialic acid 

Fucose 

KDO 

Probe	Molecular structure	Probe	Molecular structure	Probe	Molecular structure	
SiaNProc	R^1= $OCH_2C{\equiv}CH$ R^2= OH	Ac4FucAz	R^1= CH_2N_3 R^2=R^3= OAc	KDO-N_3	R^1= N^3	(Sawa et al., 2006)
Neu5Hex	R^1= $(CH_2)_3C{\equiv}CH$ R^2= OH	FucAl or 6-alkynyl Ac4fucose	R^1= C≡CH R^2= Ac R^3= Ac	9AI-KDO	R^1= C≡CH	(Homann et al., 2010; Patterson et al., 2012)
9-Cp-NeuAc	R^1= CH_3 R^2= 	FucAz	R^1= CH_2N_3 R^2= GDP	**KDO-HMMPO**	R^1= 	(Patterson et al., 2012; Sawa et al., 2006; Sherratt et al., 2014)
SiaNAl	R^1= $(CH_2)_2C{\equiv}CH$ R^2= OH		R^3= H	KDO-alkyne	R^1= $NHCO(CH_2)_2C{\equiv}CH$	(Wang et al., 2015)
9BA-Neu5Ac	R^1= OH R^2= $NHCH_2COC_6H_4COH$					(Zeng et al., 2009)
Xylose 		**Trehalose** 				(Swarts et al., 2012)
UDP-2-XylAz	R^1=UDP, R^2=N_3, R^3=OAc, R^4=OAc	2-TreAz	R^1= N_3, R^2= OH, R^3= OH, R^4= OH			
UDP-3-XylAz	R^1= UDP, R^2= OH, R^3= N3, R^4= OH	3-TreAz	R^1= OH, R^2= N_3, R^3= OH, R^4= OH			

UDP-3-XylAz	R^1= UDP, R^2=OH, R^3=OH, R^4=N_3	4-TreAz	R^1= OH, R^2= OH, R^3= N_3, R^4= OH
		6-TreAz	R^1= OH, R^2= OH, R^3= OH, R^4= N_3

Abbreviations: **1,3,4-*O*-Bu$_3$ManNAz**, 1,3,4-tri-*O*-butanoyl-*N*-azido-mannosamine; **9AI-KDO**, 9-Alkynylate-3-Deoxy-d-manno-oct-2-ulosonic acid; **9BA-Neu5Ac**, 9-benzaldehyde-*N*-acetyl-neuramic acid; **9-Cp-NeuAc**, 9-cyclopropeneacetyl-*N*-neuramic acid; **Ac$_3$-4-azido-ManNAc**, *N*-acetyl-(1,3,6-O-acetyl)-4-azido-4-deoxy-mannosamine; **Ac$_4$FucAz**, per-acetylated azide-fucose; **Ac$_4$GalDiaz**, tetra-O-acetyl-N-diazoacetylgalactosamine; **Ac$_4$GalNAlk**, *N*-(4-pentynoyl)-galactosamine-tetraacylated; **Ac$_4$GalNAz**, *N*-azidoacetylgalactosamine-tetraacylated; **Ac$_4$GalNCyoc**, *N*-Cyclopropeneacetylatedgalactosamine-tetraacylated; **Ac$_4$GalN-n-Iso**, tetra-O-acetyl-N-(3-isocyanopropanoyl)-D-galactosamine; **Ac$_4$GalN-t-Iso**, tetra-O-acetyl-N-(2-isocyano2-methylpropanoyl)-D-galactosamine; **Ac$_4$GalPoc**, *N*-propargylcarbamate-1,3,4,6-tetra-O-acetyl-galactosamine; **Ac$_4$GlcNAlk**, *N*-(4-pentynoyl)-glucosamine-tetraacylated; **Ac$_4$GlcNAloc**, *N*-(Allyloxycarbonyl)glucosamine-tetraacylated; **Ac$_4$GlcNBeoc**, *N*-(But-3-en-1-yl-oxycarbonyl)glucosamine-tetraacylated; **Ac$_4$GlcNDAz(3Me)**, 1,3,4,6-tetra-O-acetyl-2-acetyl-N-5,5-azo-hexamido-2-deoxy-α,β-D-glucopyranose; **Ac$_4$GlcNDAz(4Me)**, 1,3,4,6-tetra-O-acetyl-2-acetyl-N-6,6-azo-septamido-2-deoxy-α,β-D-glucopyranose; **Ac$_4$GlcNHeoc**, *N*-(Hex-5-en-1-yl-oxycarbonyl)glucosamine-tetraacylated; **Ac$_4$GlcN-n-Iso**, tetra-O-acetyl-N-(3-isocyanopropanoyl)-D-glucosamine; **Ac$_4$GlcNPeoc**, *N*-(Pent-4-en-1-yl-oxycarbonyl)-glucosamine-tetraacylated; **Ac$_4$GlcN-t-Iso**, tetra-O-acetyl-N-(2-isocyano2-methylpropanoyl)-D-glucosamine; **Ac$_4$GlcPoc**, *N*-propargylcarbamate-1,3,4,6-tetra-O-acetyl-glucosamine; **Ac$_4$ManN2Azaryl**, 2-Para-azidophenylacetamido-2-deoxy-α,β-D-mannopyranose-tetraacylated; **Ac$_4$ManNAlk**, *N*-(4-pentynoyl)-mannosamine-tetraacylated; **Ac$_4$ManNAloc**, *N*-(Allyloxycarbonyl)mannosamine-tetraacylated; **Ac$_4$ManNAz**, *N*-azidoacetylmannosamine-tetraacylated; **Ac$_4$ManNBeoc**, *N*-(But-3-en-1-yl-oxycarbonyl)mannosamine-tetraacylated; **Ac$_4$ManNCyc**, *N*-cyclopropeneacetylmannosamine; **Ac$_4$ManNCyoc**, *N*-Cyclopropeneacetylatedmannosamine-tetraacylated; **Ac$_4$ManNDAz(2Me)**, 1,3,4,6-tetra-O-acetyl-2-acetyl-N-4,4-azo-pentamido-2-deoxy-α,β-Dmannopyranose; **Ac$_4$ManNDAz(3Me)**, 1,3,4,6-tetra-O-acetyl-2-acetyl-N-5,5-azo-hexamido-2-deoxy-α,β-D-mannopyranose; **Ac$_4$ManNDAz(4Me)**, 1,3,4,6-tetra-O-acetyl-2-acetyl-N-6,6-azo-septamido-2-deoxy-α,β-D-mannopyranose; **Ac$_4$ManNHeoc**, *N*-(Hex-5-en-1-yl-oxycarbonyl)mannosamine-tetraacylated; **Ac$_4$ManNHxl**, *N*-(Hex-5-enoyl)mannosamine-tetraacylated; **Ac$_4$ManN-n-Iso**, tetra-O-acetyl-N-(3-isocyanopropanoyl)-D-mannosamine; **Ac$_4$ManNNorboc$_{endo}$**, 1,3,4,6-Tetra-O-acetyl-2-[(endo-bicyclo[2.2.1]hept-5-en-2-yl)methoxycarbonylamino]-2-deoxymannopyranose; **Ac$_4$ManNNorboc$_{exo}$**, 1,3,4,6-Tetra-O-acetyl-2-[(exo-bicyclo[2.2.1]hept-5-en-2-yl)methoxycarbonylamino]-2-deoxymannopyranose; **Ac$_4$ManNPeoc**, *N*-(Pent-4-en-1-yl-oxycarbonyl)-mannosamine-tetraacylated; **Ac$_4$ManNPtl**, 2-((But-3-enoyl)amino)-2-desoxy-d-mannopyranose-tetraacylated; **Ac$_4$ManN-t-Iso**, tetra-O-acetyl-N-(2-isocyano2-methylpropanoyl)-D-mannosamine; **Ac$_4$ManPoc**, *N*-propargylcarbamate1,3,4,6-tetra-O-acetyl-mannosamine; **FucAl**, Alkynylated fucose; **FucAz**, 6-azidofucose; GalNAc, *N*-acetylgalactosamine; GalNLev, *N*-levulinoyl galactosamine; GalNPro, *N*-propanoylgalactosamine; GlcNAc, *N*-acetylglucosamine; **GlcNAz**, *N*-azidoacetylglucosamine tetraacylated; **GlcNLev**, *N*-levulinoyl glucosamine; **GlcNPro**, *N*-propanoylglucosamine; **Kdo-alkyne**, 3-Deoxy-d-manno-oct-2-ulosonic acid-alkyne; **Kdo-HMMPO**, [2-(hydroxymethyl)-2-methyl-3,4-dihydro-2H-pyrrole 1-oxide]; **KDO-N$_3$**, 8-azido-3,8-dideoxy-D-manno-octulosonate; **ManNAc**, *N*-acetylmannosamine; **ManNAz**, *N*-azidoacetylmannosamine tetraacylated; **ManNLev**, *N*-Levulinoyl mannosamine; **ManNPro**, *N*-propanoylmannosamine; **Neu5Hex**, *N*-(1-oxohex-5-ynyl) neuraminic acid; **SiaNAl**, *N*-(4-pentynoyl)neuraminic acid; **SiaNProc**, *N*-Cyclopropanoylsialidase; **UDP-2-XylAz**, UDP-2-azido-4-deoxyxylose; **UDP-3-XylAz**, UDP-3-azido-4-deoxyxylose; **UDP-4-XylAz**, UDP-4-azido-4-deoxyxylose.

Table 2. Various applications of bacterial-based enginnered glycans

Bacteria	Glycan	Modification	Application	Ref.
Aureobasidium melanogenum P16	Pullulan	Inulinase gene transfer from *Kluyveromyces marxianus* KM into *A. melanogenum* (P16)	Food, chemical, cosmetics, agricultural and pharmaceutical industries	(Ma et al., 2015)
X. campestris Xc17L	Xanthan gum	Improve the β-galactosidase activity	food industry, pharmaceutical industries, agriculture products	(Yang et al., 2002)
Agrobacterium sp. ATCC31749	Curdlan	Curdlan biosynthesis operon (*crd*) genes overexpression	Thickener, texturizer and stabilizer in the food industries	(Yu et al., 2015)
P. aruginosa	Alginate		industrial and pharmaceutical	(Maerk et al., 2020)
Azotobacter vinelandii ATCC12518Tc		Regulatory proteins, AlgW and AmrZ		
Lactococcus lactis	EPS	Overexpression of UDP-glucose pyrophosphorylase (GalU)	Prebiotic nutrition, for food texture and taste improvement	(Boels et al., 2001; Levander et al., 2002)
Streptococcus thermophilus		Phosphoglucomutase gene (*pgm*)		
Bordetella pertussis	LPS	*pagP* and *pagL* (LPS modification genes)	Live attenuated vaccine (Increased protection against	(Beceiro et al., 2014;

			B. pertussis)	Rolin et al., 2014)
E. coli BL21	Carbapenem	Overexpression of carbapenem production genes (car gene cluster, carD, carE and carB)	B-lactum antibiotic	(Shomar et al., 2018)
E. coli K12	Isoflavonoid-7-*O*-methoxides	Genetically engineered *metK* for encoding *S*-adenosylmethionine (SAM) synthase	Anticancer	(Koirala et al., 2019)
B. licheniformis DW2	Bacitracin	Enhanced lysine (precursor) supply via metabolic engineering	Antibacterial	(Wu et al., 2019)
E. coli	Human milk oligosaccharides	lgtA encodes β-1,3-Nacetylglucosaminyltransferase was introduced in host strain	Nutraceutical	(Zhu et al., 2021)
E. coli	Colanic acid	Byproduct genes and 11 O-antigen production genes were deleted. Lipid carrier gene uppS was overexpressed	Food and healthcare	(Zhan et al., 2022)
Pichia pastoris	Chondroitin sulfate A	kfoC, kfoA, tuaD, C4OST expression, overexpression of endogenous genes coding ATPS and APSK	dietary supplement for osteoarthritis	(Jin et al., 2021)
E. coli C2987		Expression of kfoA, kfoC, kfoF and vgb		(Erenler, 2019)
E. coli O5:K4:H4	Fructosylated chondroitin,	Overexpression of kfoA and kfoF		(Awofiranye et al., 2020; Cimini et al., 2018)
	N-glycolyl chondroitin	kfoE deletion		
E. coli 126E	Globotriose, globotetraose	Overexpressing s lgtC gene for a-1,4-Gal transferase, isolated from *N. meningitides*	affinity inhibitors for the toxins in the prevention and treatment of related diseases	(Antoine, T. et al., 2005)

E. coli mutant DJ	Nonsulfated HNK-1 carbohydrate	Mouse glucuronyl transferase (GlcAT-P) was cloned and expressed in an engineered *E. coli* strain	Immunological applications	(Yavuz et al., 2008)
E. coli	Resveratrol	Stilbene synthase modification and enhanced intracellular malonyl-CoA supply	Polyphenol, nutritional compound	(Liu et al., 2017)
E. coli	Hyaluronic acid	Multiple genes expression manipulation and introduction of hyaluronic acid synthase gene *hasA* from *Streptococcus zooepidemicus*	medical and cosmetic applications	(Woo et al., 2019)