Engineered production of bioactive polyphenolic *O*-glycosides

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ABSTRACT

Polyphenolic compounds (such as quercetin and resveratrol) possess potential medicinal values due to their various bioactivities, but poor water solubility hinders their health benefits to humankind. Glycosylation is a well-known post-modification method to biosynthesize natural product glycosides with improved hydrophilicity. Glycosylation has profound effects on decreasing toxicity, increasing bioavailability and stability, together with changing bioactivity of polyphenolic compounds. Therefore, polyphenolic glycosides can be used as food additives, therapeutics, and nutraceuticals. Engineered biosynthesis provides an environmentally friendly and cost-effective approach to generate polyphenolic glycosides through the use of various glycosyltransferases (GTs) and sugar biosynthetic enzymes. GTs transfer the sugar moieties from nucleotide-activated diphosphate sugar (NDP-sugar) donors to sugar acceptors such as polyphenolic compounds. In this review, we systematically review and summarize the representative polyphenolic *O*-glycosides with various bioactivities and their engineered biosynthesis in microbes with different biotechnological strategies. We also review the major routes towards NDP-sugar formation in microbes, which is significant for producing unusual or novel glycosides. Finally, we discuss the trends in NDP-sugar based glycosylation research to promote the development of prodrugs that positively impact human health and wellness.

Keywords:

Polyphenolic compounds

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Bioactivities

Bioavailability

Glycosylation

Glycosyltransferase

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1. Introduction

Scientists, nutritionists, food manufacturers, and consumers have great interest in dietary polyphenols due to their enormous health benefits to humans. Polyphenols are often acquired from dietary sources including fruits, beverages (fruit juice, wine, tea, coffee, and beer), vegetables, whole grains, soy products, dry legumes and cereals (Scalbert and Williamson, 2000). In recent years, extensive epidemiological research studies have shown that consumption of a polyphenol-rich diet can prevent humans from developing various degenerative and chronic diseases, including cancers (Ferrazzano et al., 2011; Yang et al., 2000), inflammation (Bowden, 1999), cardiovascular (atherosclerosis) and neurodegenerative diseases (McSweeney and Seetharaman, 2015), liver disorder (Bose et al., 2008), obesity (Lu et al., 2012), diabetes (Scalbert et al., 2005), aging (Cherniack, 2010), and infectious diseases (Rasouli et al., 2017). The broad spectrum of bioactivities within the polyphenol family makes these compounds excellent targets for the development of potential medicines and nutraceuticals (Yang et al., 2018). Most of the aforementioned diseases are related to oxidative stress from reactive oxygen and nitrogen species (Duthie and Brown, 1994; Goldberg and Hasler, 1996; Tsao, 2010). Moreover, polyphenols have antibacterial and antifungal activities (Papuc et al., 2017), which can be used as natural preservatives for the meat industry (Ferrazzano et al., 2011). Polyphenols are reducing agents with strong antioxidant activities that can work together with other dietary reducing agents, such as vitamin C, vitamin E and carotenoids to protect body tissues from excess reactive oxygen species (ROS) (Williams et al., 2004). Furthermore, in terms of their chemical

nature, polyphenols are the most abundant antioxidants in our daily diets, reducing oxidative damage to lipids, proteins, enzymes, carbohydrates, and DNA in living cells and tissues (Cirillo et al., 2016).

Polyphenols are a large group of natural compounds with one or more hydroxyl groups attached to the phenyl ring, and some of them occur as glycosides in nature (Quideau et al., 2011). At present, over 8,000 phenolic structures have been identified, and they are a collective term for several sub-groups of phenolic compounds. Based on the chemical structures, polyphenols can mainly be divided into six groups, including flavonoids, phenolic compounds, stilbenes, curcuminoids, lignans, and polyphenolic amides (Tsao, 2010). Among them, flavonoids and phenolic compounds account for two thirds and nearly the remaining one third of the total dietary polyphenol intake, respectively. Studies have shown that new flavonoids and their glycosides continue to be identified from nature (Hu et al., 2022; Jiang et al., 2022; Suleimen et al., 2022; Veitch and Grayer, 2008; Wu et al., 2022; Zhong et al., 2022). Flavanols, including proanthocyanidins, anthocyanins and their oxidation products, are the most abundant flavonoids in the human diet (Scalbert and Williamson, 2000). As the secondary metabolites produced by plants, polyphenols can not only help the growth and development of the plants, but also act as phytoalexin to protect themselves against biotic stress resulting from other organisms such as bacteria, viruses, fungi, and herbivores (Rasouli et al., 2016; Yang et al., 2018).

In addition to the diverse health benefits for humankind, there are many other advantages of polyphenols, including easy accessibility, high response specificity, and

low toxicity. However, rapid metabolism and low bioavailability of polyphenols are non-negligible problems (Rasouli et al., 2017; Suresh and Nangia, 2018). Attaching a glycosidic moiety to the polyphenols, called glycosylation, can not only provide a structurally diverse pool of flavonoids, but also provides a simple and effective way to improve their water solubility and stability (Yang et al., 2018). The change of hydrophilicity can further influence the pharmacokinetic properties of the respective compounds, including circulation, elimination and concentration regulation in body fluids (Kren and Martínková, 2001). Therefore, glycosylation can modulate polyphenol bioactivities, bioavailability and, in some cases, even their color and taste. The bioactivity and bioavailability of flavonoid glycosides *in vivo* are often related to each other (Arbeláez et al., 2015; Zhang et al., 2014). Glycoconjugates also have other functions, including information storage and transfer, energy storage, maintenance of cell structural integrity, molecular recognition, signaling, virulence, and chemical defense (Thibodeaux et al., 2008). Researchers have proved the importance of sugar moieties in bioactive natural products (Weymouth-Wilson, 1997), including various antibiotics (Luzhetskyy et al., 2005). Phenolic hydroxyl groups are generally good targets for biological glycosylation, and some biologically active polyphenols only occur naturally in their glycosylated forms. Polyphenol glycosides are shown to possess various biological activities, such as antioxidant, immunomodulatory, and anticancer activities (Kim et al., 2015; La Ferla et al., 2011).

Glycosylation can change the chemical and physical properties of polyphenols, which helps the stabilization, enhancement of water solubility, and detoxification of

polyphenols. In the past two decades, many researchers have tried to harness the power of biological catalysts to modify the sugar structures and glycosylation patterns of polyphenolic natural products both *in vivo* and *in vitro*. With the help of bioengineering methodology together with biochemical and structural studies of sugar biosynthetic enzymes and GTs, the development of more effective and even novel glyco-drugs development has been facilitated. In this review, we summarize some of the most common bioactive phenolic glycosides and biological approaches to produce them. This review will not only shed light on the diverse combinatorial biosynthetic mechanisms and natural product evolution in bacteria, but also provide various methods to manipulate sugar biosynthetic machinery for generating clinically useful agents.

2. Classification of polyphenolic compounds, functions of glycosylation, and production approaches of polyphenolic glycosides

Polyphenolic compounds feature one or more aromatic rings, with hydroxyl groups attached to various positions of the core structures. Based on their parent structures, polyphenolic compounds can be mainly classified into two groups: flavonoids and non-flavonoids. Flavonoids have a general 15-carbon skeleton structure with C_6-C_3-C_6 backbone featuring the two phenyl rings, namely ring A and ring B. Flavonoids represent more than 50% of total polyphenolic compounds Due to the hydroxylation pattern and variations in the oxygenated heterocyclic ring C, flavonoids can be further divided into nine different sub-groups, including flavonols, flavones, flavanones, flavononols, flavanols, isoflavones, anthocyanidins, chalcones, and

neoflavones. Flavanols can be further condensed into its corresponding polymers, namely procyanidins. Non-flavonoids contain phenolic acids (including hydroxybenzoic acids and hydroxycinnamic acids), stilbenes, curcuminoids, lignans (including neolignans), polyphenolic amides and others (Fig. 1) (Rasouli et al., 2017).

Fig. 1

Despite the diverse chemical reservoir of polyphenolic compounds, low bioavailability hampers their health benefits to humans. Glycosylation of polyphenolic compounds is a promising approach to increase their water solubility and further modulate their biological effects (Bashir et al., 2020). The glycosylation process of small molecules is catalyzed by carbohydrate-active enzymes which are generally divided into four types for *in vitro* glycosylation of polyphenolic compounds (Fig. 2a): (1) transglycosidases (TGs) transfer the sugar moiety from non-activated sugar (such as sucrose) to small molecules (Li, X. et al., 2021); (2) glycoside hydrolases (GHs) are hydrolytic enzymes for biosynthesizing polyphenolic glycosides (De Winter et al., 2013); (3) glycoside phosphorylases (GPs) require glycosyl phosphates (such as glucose-1-phosphate) as donors (Kwon et al., 2007); (4) glycosyltransferases (GTs) transfer sugar from a donor (such as lipid phosphate sugar, phosphate sugars, and nucleotide-activated sugars) to an acceptor molecule for glycosylation (Breton et al., 2012; Moremen and Haltiwanger, 2019). Most of GTs are Leloir-type GTs which use nucleotide-activated sugars (such as UDP-glucose) as donors (Xu et al., 2022).

Considering conversion efficiency, affinity to various substrates, concentration requirement of aglycons, and theirs diversity, the GT family is the most practical of

these four for the *in vivo* engineered production of polyphenolic glycosides (De Winter et al., 2014; Desmet et al., 2012). Glycosylation reactions are mainly catalyzed by nucleotide sugar-dependent family 1 GTs (Vogt and Jones, 2000), which can transfer the sugar moiety from an activated sugar donor (such as UDP- or dTDP-sugars) to the acceptors. One common type of sugar acceptors are small molecules, such as the polyphenolic compounds detailed in this review (Fig. 2b) (De Bruyn et al., 2015b). Since the first GT from *Zea mays* was found in 1984 (Fedoroff et al., 1984), many researchers have devoted time to searching various GTs from plants or microorganisms. GTs can be classified as *O*-, *C*-, *N*-, or *S*-GTs, with *O*-GTs being the most abundant in nature (Ati et al., 2017; Putkaradze et al., 2021).

Glycosylation of polyphenolic compounds can decrease their toxicity as well as alter their bioavailability, bioactivity, stability, and/or other properties (Xiao, 2017). Remarkable examples are the glycosylation of quercetin for improved stability (Buchner et al., 2006), rhamnosylation of kaempferol for unique activities such as diuretic and renal protective effects (Cechinel-Zanchett et al., 2020), glucosylation of resveratrol as well as glucuronylation and rhamnosylation of 2'-hydroxyflavone for higher antioxidant activity (Ren et al., 2022b; Su et al., 2013), glucosylation of vanillin and hydroquinone for decreased toxicity (Chandorkar et al., 2021; Hansen et al., 2009), and glucosylation and rhamnosylation of quercetin for improved bioavailability (Fig. 2c) (Valentová et al., 2014; Wagner et al., 2006).

Glycosylation is a promising approach to develop new therapeutic agents by improving bioavailability of polyphenolic compounds which may further modulate

their biological activities (Costa et al., 2020; Navarro-Orcajada et al., 2022; Zhao et al., 2019). Polyphenolic glycosides normally keep higher plasma levels and have a longer mean residence time than those of aglycones (Jiang et al., 2008; Zhang et al., 2013). Therefore, glycosides could be considered and used as pro-drugs with improved water solubility (Walle et al., 2005). Polyphenolic glycosides cannot diffuse across the cellular membrane due to high water solubility, low permeability, and larger molecular weight (Zhang et al., 2005), but their hydrophobic aglycones after hydrolyzation can be easily absorbed by the epithelial cells through passive diffusion (Chen et al., 2011). Human small intestine and large intestinal colonic microflora are important for hydrolyzation, with various enzymes such as β-glucosidase, β-glucuronidase, and α-rhamnosidase (Arts et al., 2004; Hur et al., 2000; Lee et al., 2011). It was shown that incubation of flavonoid glycosides with feces can generate corresponding aglycones (Hanske et al., 2009). After absorption in small intestine, flavonoids will be metabolized into their glucuronide or sulfate conjugates by phase II enzymes and then be bound to albumin and transported to the liver via the portal vein (Murakami et al., 2008; Xiao and Kai, 2012). Flavonoids undergo hydroxylation, methylation, reduction, sulfation and glucuronidation to form various flavonoid metabolites in the liver (Fig. 2d) (Xiao and Hogger, 2013).

Fig. 2

Due to their wide-reaching importance, different methods have been exploited to acquire polyphenolic glycosides. However, many production methods of plant polyphenol glycosides are not environmentally friendly and difficult to apply in

231 industry for scale-up. Extraction suffers from low production yield, and the plant
232 extraction processes is not only tedious and time-consuming, but harmful to the
233 environment and human health (Sordon et al., 2016). Chemical synthesis is complicated
234 with many protection, activation, and deprotection steps required (Yang and Yu, 2017).
235 Moreover, extreme temperatures, high pressure, and the use of organic solvents and
236 various uncommon solid chemicals (Yang et al., 2015) render this approach neither
237 sustainable nor economically friendly (Orsini et al., 1997). Enzymatic synthesis needs
238 expensive cofactors and additional substrates to perform the *in vitro* enzymatic
239 reactions (Zheng et al., 2022), and nucleoside diphosphate sugar donors have limited
240 availability with high costs (Gantt et al., 2011). Because tedious purification processes
241 are required to obtain pure enzymes and final product yield tends to be very low, large-
242 scale production is almost unachievable through this route (Marié et al., 2018).
243 Compared with these methods, engineered biosynthesis is more applicable for
244 industrial production of medicinally important polyphenolic glycosides (Fig. 3).

245 **Fig. 3**

246 The engineered biosynthesis approach has many advantages over the
247 aforementioned methods, including high yield, low cost, high efficiency, easy operation
248 and environmentally-friendly processing (Fig. 3) (Yang et al., 2018). Moreover, this
249 method can also synthesize uncommon NDP-sugars that are not commercially available
250 for generating novel products (Thibodeaux et al., 2007). Finally, uridine diphosphate
251 glycosyltransferases (UGTs) from plants normally show both sugar-donor and sugar-
252 acceptor selectivity, so regioselectivity is an important advantage of engineered

biosynthesis using UGT-expressing bacteria. Since metabolic engineering of plants is controversial and not fully developed (Verpoorte and Memelink, 2002), biosynthesis of valuable glycosides is mainly focused on the use of engineered microorganisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*. Due to the attractive properties of polyphenolic *O*-glycosides and their diversity in nature, engineered production of bioactive polyphenolic *O*-glycosides is summarized in this review.

3. Microbial production of bioactive polyphenolic *O*-glycosides

Polyphenolic glycosides have diverse biological activities, and remarkable examples include quercetin glycosides with antioxidant (Nile et al., 2017), antiasthmatic (Zhu et al., 2019), and wound-healing activities (Özbilgin et al., 2018). Moreover, quercetin glycosides also exert a protective effect on dexamethasone-induced muscle atrophy (Otsuka et al., 2019) and obesity (Jiang et al., 2020). However, the low concentration of most polyphenolic glycosides in plants despite their widespread distribution in nature presents a challenge for their production. Microbial biosynthesis is a promising approach to produce glycosides, but the production efficiency can be low due to insufficient availability of UDP-sugars (Feng et al., 2020). By applying metabolic engineering, protein engineering, fermentation engineering, and synthetic biology approaches, both natural and unnatural polyphenolic glycosides can be produced successfully in microbes with improved production titers. Polyphenolic glycosides are versatile resources for investigating biological activities, and those with significant bioactivities can be developed into medicines, functional foods, and even cosmetics. Glycosyltransferases (GTs) found in plants and microorganisms are

commonly used to glycosylate polyphenolic compounds. The types, numbers, and positions of the sugar moieties on the aglycones contribute to the diversity of polyphenolic glycosides. As a result, numerous examples of successful production of *O*-glycosides have been achieved through engineering.

3.1. Engineered production of flavonol glycosides

3.1.1. Quercetin-3-O-glucoside (isoquercetin)

Flavonols (such as quercetin, kaempferol, myricitrin, and fisetin) have a double bond between C-2 and C-3, and a hydroxy group attached at C-3. Flavonols are frequently glycosylated with various sugar moieties, such as glucose, glucuronic acid, rhamnose, galactose, xylose, and so on (Zhang et al., 2006). Quercetin-3-*O*-glucoside (isoquercetin) is a well-known plant secondary product with strong antioxidant (Razavi et al., 2009) and neuroprotective effects (Yang et al., 2021). Isoquercetin is the main component of *Annona squamosa* leaves for its antidiabetic and antioxidative effects (Panda and Kar, 2007). To achieve the engineered production of isoquercetin, Xia engineered a single gene deletion strain *E. coli* MEC367/Δ*pgi* expressing UGT73B3 from *A. thaliana*, and production titer was improved in 1-L controlled bioreactors by providing a higher oxygen transfer rate. Finally, the production titer of isoquercetin reached 3.9 g/L in 56 h with 30 g/L glucose as the sole carbon source and 5 g/L quercetin as the substrate (Xia and Eiteman, 2017). This study indicates that compared to shaker flasks, bioreactors are an effective method for improving the production titer because of the improvement in culture oxygenation. Some researchers have worked on glucosyltransferases from other resources. Ren et al. achieved the engineered

297 production of isoquercetin at 99 mg/L from 125 mg/L quercetin with 20 g/L glucose by
298 introducing a glucosyltransferase from *Beauveria bassiana* ATCC 7159 into *S.*
299 *cerevisiae* and optimizing the fermentation factors (Ren et al., 2022c), demonstrating
300 that yeast can also be used as the host for engineered production of isoquercetin, in
301 addition to *E. coli.*

302 *3.1.2.* Quercetin-3-*O*-glucuronide (miquelianin)

303 Quercetin-3-O-glucuronide (miquelianin) has a variety of health benefits,
304 including anti-inflammatory effects (Derlindati et al., 2012), protective effects against
305 neurotoxicity (Pariyar et al., 2019), and anti-breast cancer properties (Yamazaki et al.,
306 2014). It also possesses antioxidant (Wu et al., 2019), antidepressant (Juergenliemk et
307 al., 2003), antimelanogenesis (Ha et al., 2021), antidiabetic (Ahmed et al., 2019), and
308 anti-Alzheimer activities (Ho et al., 2013). However, engineered production of
309 miquelianin is hindered by insufficient production of UDP-glucuronic acid in the hosts.
310 To address this issue, Kim et al. deleted the *araA* gene in *E. coli* which encodes both
311 UDP-4-deoxy-4-formamido-L-arabinose formyltransferase and UDP-glucuronic acid
312 C-4" decarboxylase, and overexpressed UDP-glucose dehydrogenase gene (*ugd*) that
313 converts UDP-glucose into UDP-glucuronic acid. They selected the *ugd* gene from *E.*
314 *coli* instead of those from A. thaliana and Glycine max because of its high efficiency.
315 In the final glycosylation step of miquelianin synthesis from quercetin in *E. coli*, Kim
316 et al. utilized VvUGT from *Vitis vinifera*, resulting in a final production of 687 mg/L
317 (Kim et al., 2015). This work demonstrates the importance of testing enzymes from
318 different origins to select the most efficient one for enhancing the final production titer

of target glycosides. In a similar study, Pandey et al. employed VvGT5 from *V. vinifera* in a single vector system with co-expressed UDP-glucuronic acid biosynthetic genes and a glucokinase gene in E. coli BL21(DE3). This approach resulted in a production titer of 30 mg/L miquelianin (Pandey et al., 2019)., representing a 31% conversion of quercetin. This study highlights the feasibility of assembling nucleotide sugar biosynthetic genes in a single vector for sufficient production of NDP-sugars.

3.1.3. Quercetin-3-O-galactoside (Hyperoside)

Quercetin-3-*O*-galactoside (hyperoside) can be isolated from various plants (Raza et al., 2017). Hyperoside has anti-inflammatory (Kim et al., 2011; Ku et al., 2015), antiviral (Wu et al., 2007), and antioxidant activities (Piao et al., 2008). One recent study showed that hyperoside has a protective effect on liver injury (Hu et al., 2020). Engineered production of hyperoside has been successfully achieved. Bruyn et al. developed an *in vivo* glycosylation platform in *E. coli* W with the record high production of 0.94 g/L hyperoside from two inexpensive substrates, namely sucrose and quercetin. To provide enough UDP-sugars, the engineered strain *E. coli* W/Δ*pgi*Δ*agp*Δ*ushA*Δ*galETKM* (galactose operon) was constructed. By overexpressing the uridylyltransferase (*ugpA*) from *Bifidobacterium bifidum*, D-glucose-1-phosphate can be efficiently channeled towards UDP-D-glucose. Then, the UDP-glucose epimerase (*galE*) from *E. coli* was introduced into the route to convert UDP-glucose into UDP-galactose. Lastly, the final *E. coli* W mutant was engineered by overexpressing the flavonol-3-*O*-galactosyltransferase (*F3GT*) from *Petunia hybrida* (De Bruyn et al., 2015c). This study demonstrates that by deleting competing pathways

responsible for synthesizing UDP-D-glucose and introducing heterologous plant GT in E. coli, large-scale preparation of flavonoid glycosides can be achieved to meet the increasing market demand. Similarly, in a study by Kim et al. (2015), 280 mg/L hyperoside was successfully synthesized in engineered *E. coli* by overexpressing UDP-glucose epimerase UGE from *Oryza sativa* and a GT called PhUGT from *Petunia hybrid* (Kim et al., 2015). Thus, it is apparent that the supply of the target nucleotide sugar through overexpression of specific NDP-sugar biosynthetic genes is crucial for producing relative glycosides.

3.1.4. Quercetin-3-O-xyloside

Research has shown that quercetin-3-*O*-xyloside has immune-stimulating properties (Lee et al., 2016), and can also ameliorate acute pancreatitis (Seo et al., 2019). Some studies have focused on manipulating xylose biosynthetic genes to produce quercetin xyloside. Pandey et al. overexpressed four genes in *E. coli* BL21(DE3) to generate a cytoplasmic pool of UDP-xylose, including phosphoglucomutase (*nfa44530*) from *Nocardia farcinica*, glucose-1-phosphate uridylyltransferase (*galU*) from *E. coli* K12, as well as UDP-glucose dehydrogenase (*calS8*) and UDP-glucuronic acid decarboxylase (*calS9*) from *Micromonospora echinospora* sp. *calichenesis*. To produce quercetin-3-O-xyloside, the researchers constructed an engineered strain *E. coli* BL21(DE3)/*ΔpgiΔzwfΔushA* with the UDP-xylose biosynthetic cassette and a GT gene (*arGt-3*) from *A. thaliana*. The maximum product concentration reached 23.78 mg/L in 5-mL culture tubes with 100 μM quercetin. When the reaction was scaled up to a 3-L fermentor, the titer reached up to 127.6 mg/L in 36 hours (Pandey et al., 2013). This

study shows the feasibility of glycosylating quercetin into its xyloside in *E. coli*, and emphasizes the importance of fermentation engineering in enhancing production titer. Deleting the competing pathway is also important for accumulating the pool of UDP-xylose. Han et al. deleted the UDP-L-Ara4N formyltransferase/UDPGlcA C-4-decarboxylase gene (*arnA*) that competes with *UXS* (UDP-xylose synthase) for UDP-glucuronic acid, and overexpressed *UXS* from *A. thaliana* and *ugd* (UDP-glucose dehydrogenase) from *E. coli*. With the aid of a GT named AtUGT78D3, they obtained approximately 160 mg/L of quercetin-3-O-xyloside in the engineered *E. coli* strain (Han et al., 2014). This study highlights that *E. coli* can synthesize not only common nucleotide sugars like UDP-glucose and dTDP-rhamnose, but also uncommon ones not synthesized in most plants, such as UDP-L-Ara4FN. Hence, flux rewiring by deleting the genes responsible for competing pathways in *E. coli* is an effective approach for increasing the production of target glycosides.

3.1.5. Quercetin-3-O-rhamnoside (Quercitrin)

Quercetin-3-*O*-rhamnoside (quercetrin) is a plant natural product with antiviral (Choi, H.J. et al., 2009), anti-hyperlipidemic (Herni et al., 2021), anticancer (Kim, D.-K. et al., 2012), anti-inflammatory, anti-oxidative (Indriyanti et al., 2018), and antileishmanial effects (Muzitano et al., 2006). Engineered production of quercetrin in microbes provides an efficient approach for its large-scale preparation. Simkhada et al. inserted two recombinant plasmids in *E. coli* BL21(DE3)/Δ*pgi* for rhamnoside production, including pCDTGSDH carrying *tgs* from *Thermus caldophilus* GK24 and *dh* from *Salmonella thyphimurium* LT2 and pAC-EPKR carrying *epi* and *kr* genes from

Streptomyces antibioticus Tü99. These genes directed the flux from glucose-1-phosphate to dTDP-L-rhamnose and led to the production of 24 mg/L quercitrin (Simkhada et al., 2010). Besides manipulating the genes in *E. coli* for supplying sufficient endogenous dTDP-L-rhamnose for glycosylation, some researchers used the plant rhamnose synthase gene to convert UDP-glucose directly into UDP-rhamnose in *E. coli* for rhamnoside biosynthesis. To prevent the production of dTDP-L-rhamnose from dTDP-4-dehydro-6-deoxy-L-mannose in *E. coli*, Kim et al. deleted the dTDP-4-dehydrorhamnose reductase (*rfbD*) to construct the mutant strain *E. coli* BrfbD. Next, they introduced rhamnose synthase (*rhm*) to generate UDP-rhamnose directly from endogenous UDP-glucose and rhamnose flavonol glycosyltransferase (*AtUGT78D1*) from *A. thaliana* into *E. coli* BrfbD. The resulting strain *E. coli* B204 produced 150 mg/L quercitrin in 48 hours (Kim, B.-G. et al., 2012a). This study provides an effective method to biosynthesize bioactive rhamnosides with fewer gene manipulation steps. To further improve the production titer of quercitrin, Bruyn et al. used the *E. coli* W/Δ*pg*Δ*agp*Δ*ushA*Δ*galETKM* glycosylation platform with the overexpression of *ugpA*, to create a pool of UDP-glucose. To convert UDP-glucose into UDP-rhamnose, they introduced the UDP-rhamnose synthase (*MUM4*) from *A. thaliana* and the flavonol-3-*O*-rhamnosyltransferase from *A. thaliana* (RhaGT) in the metabolically engineered *E. coli* W mutant. Eventually, 1.12 g/L quercitrin was biosynthesized from 1.5 g/L of quercetin in 16 hours (De Bruyn et al., 2015c). This study demonstrates the potential for selecting specific *E. coli* strains to produce certain glycosides. For instance, *E. coli* BL21(DE3) is capable of producing endogenous dTDP-rhamnose, while *E. coli* W

lacks the *rfb* gene cluster necessary for synthesizing dTDP-rhamnose, making it unable to provide this rhamnose donor for rhamnosylation. Therefore, a thorough understanding of endogenous pathways in the host strains is important for engineered production of quercetrin. It should be noted that endogenous dTDP-rhamnose is not the only sugar donor for the biosynthesis of rhamnosides; UDP-rhamnose, produced by plant UDP-rhamnose synthase, can also serve as an efficient sugar donor.

3.1.6. Quercetin-3,7-O-bisrhamnoside and quercetin 3-O-glucoside-7-O-rhamnoside

Recent research has shown that quercetin bisrhamnosides possess antiviral properties, leading to a surge of interest in exploring other quercetin bisglycosides (Choi et al., 2018; Choi, et al., 2009). Isolation of quercetin bisrhamnoside and similar products from plants is challenging due to their presence in intricate mixtures (Scognamiglio et al., 2016). Given the presence of different hydroxyl groups in flavonoids, researchers managed to biosynthesize bisglycosides by expressing two regio-specific GTs sequentially. To synthesize quercetin-3,7-*O*-bisrhamnoside, Kim et al. first used AtUGT78D1 to attach a rhamnose moiety to the 3-OH group of quercetin, and then AtUGT89C1 was employed to attach rhamnose at the 7-OH. Both GTs were from *A. thaliana*. Furthermore, the *RHM2* gene from *A. thaliana* was expressed to create sufficient UDP-rhamnose from UDP-glucose. In this work, 67.4 mg/L of quercetin-3,7-*O*-bisrhamnoside was produced by engineered *E. coli* (Kim et al., 2013). Similarly, by using a flavonol-3-*O*-glucosyltransferase AtUGT78D2 and a flavonol-7-*O*-rhamnosyltransferase AtUGT89C1 from *A. thaliana*, 67 mg/L of quercetin-3-*O*-glucoside-7-*O*-rhamnoside was produced from quercetin (Kim et al., 2013). These

studies illustrate that *E. coli* can also be used as a platform strain to synthesize flavonoid diglycosides by expressing two GTs sequentially. The regioselectivity of the second GT is crucial for forming diglycosides from monoglycosides by attaching a sugar moiety to a different hydroxy group of phenolic compounds.

3.1.7. Quercetin-3-O-alloside

The biosynthetic pathway of quercetin alloside is relatively longer compared to many other quercetin glycosides. To synthesize dTDP-6-deoxy-D-allose, which serves as a donor of allose, Simkhada et al. constructed two recombinant plasmids. The first plasmid is pCDTGSDH which contains *tgs* from *Thermus caldophilus* GK24 and *dh* from *Salmonella thyphimurium* LT2, diverting D-glucose-1-phosphate into thymidine diphosphate 4-keto 4,6-dideoxy-D-glucose (dTKDG). The second plasmid is pAC-GerFK harboring a bifunctional-gene *GerFK* from *Streptomyces* sp. KCTC 0041BP which encodes both dTDP-hexose-3-epimerase and dTDP-4-keto-6-deoxyglucose reductase, directing the pathway from dTKDG towards dTDP-6-deoxy-D-allose. These two plasmids were transferred into *E. coli* BL21 (DE3)/Δ*pgi* to yield *E. coli* BL21 (DE3)/Δ*pgi*/pCDTGSDH/pAC-GerFK. The resulting strain was able to produce dTDP-6-deoxy-D-allose, which was further used as a sugar donor for flavonoid glycosylation. The GT gene (*arGt-3*) from *A. thaliana* was simultaneously overexpressed to form quercetin-3-*O*-alloside (Simkhada et al., 2010). This study clearly reveals that engineering nucleotide sugar pathways is effective in producing quercetin alloside.

3.1.8. Quercetin-3-O-taloside

Another notable example of an engineered flavonoid glycoside is quercetin-3-*O*-

taloside, which has been successfully produced in *E. coli*. Yoon et al. introduced gene *tll* from *Actinobacillus actinomycetemcomitans* that encodes dTDP-6-deoxy-L-lyxo-4-hexulose reductase, converting the endogenous nucleotide sugar dTDP-4-dehydro-6-deoxy-L-mannose to dTDP-6-deoxy-L-talose. To increase the production of dTDP-6-deoxy-L-talose in *E. coli*, they constructed the mutant strain *E. coli*/Δ*galU*Δ*rffA*Δ*rfbD*. After conducting molecular modeling analysis, the researchers selected AtUGT78D1 from *A. thaliana* to use dTDP-6-deoxy-L-talose. Ultimately, the engineered E. coli strain produced approximately 98 mg/L of quercetin-3-*O*-taloside (Yoon et al., 2012). This study demonstrates that unwanted by-products, such as quercetin-3-*O*-glucose and quercetin-3-*O*-rhamnose, can be reduced by blocking their relative pathways, even though it is nearly impossible to quantify each nucleotide sugar in the cells. Additionally, the researchers found that molecular docking analysis of GT using both uncommon nucleotide sugar (dTDP-6-deoxy-L-talose) and common nucleotide sugar (dTDP-L-rhamnose) is useful for predicting the glycosylation products. Overall, this study highlights that the intracellular concentration of different nucleotide sugars can be manipulated by regulating the nucleotide sugar biosynthetic pathways, thus reducing the supply of unwanted nucleotide sugars and increasing the production of target glycosides.

3.1.9. Quercetin-3-O-4-deoxy-4-formamido-L-arabinoside

Naturally occurring flavonoid sugar conjugates include glucoside, galactoside, glucuronide, rhamnoside, xyloside, and arabinoside as mentioned above. Flavonoid deoxyaminosugar conjugates have not been found in nature. Kim et al. synthesized the

unusual deoxyaminosugar, UDP-4-deoxy-4-formamido-L-arabinose (UDP-L-Ara4FN) by overexpressing three genes. First, UDP-glucose was converted into UDP-glucuronic acid by *ugd*, and then *arnA* encoding both UDP-L-Ara4N formyltransferase/UDP-GlcA C-4"-decarboxylase converts UDP-glucuronic acid into UDP-4"-ketopentose. Next, *arnB* encoding UDP-L-Ara4O C-4" transaminase transfers an amino group to form UDP-4-amino-4-deoxy-L-arabinose (UDP-Ara4N). Finally, *ArnA* further converts UDP-Ara4N to UDP-4-deoxy-4-formamido-L-arabinose (UDP-L-Ara4FN) which serves as the sugar donor for the synthesis of quercetin-3-*O*-Ara4FN by AtUGT78D3 from *A. thaliana* with the final yield of around 70 mg/L (Kim et al., 2010). This study provides a promising way to biosynthesize uncommon or unnatural flavonoid glycosides by rationally engineering unusual nucleotide sugar biosynthetic pathways into *E. coli*.

3.1.10. Quercetin deoxyaminosides

Many bioactive natural products from microbes contain deoxyaminosugar moieties (Elshahawi et al., 2015), including doxorubicin, amphotericin B, erythromycin, vancomycin, and staurosporine. Deoxyaminosugars play important roles in the biological activities of these compounds (Křen and Řezanka, 2008). In addition to improving water solubility, they can alter the basicity of the compounds, which can then change their mechanism of action, such as ionic interactions (Pedersen et al., 2011). Thus, conjugation of deoxyaminosugars is useful for improving pharmacological properties of natural products.

To date, quercetin deoxyaminosides have been successfully biosynthesized using

495 engineered *E. coli*. Pandey et al. first developed a background strain *E. coli*
496 BL21(DE3)/Δ*pgi*Δ*zwf*Δ*galU* to direct the flux from glucose to glucose-1-phosphate.
497 They then converted glucose-1-phosphate to dTKDG by overexpressing *tgs* and *dh*.
498 Four sugar aminotransferase genes, including *gerB* from *Streptomyces* sp. GERI-155,
499 *wecE* from *E. coli* K-12, together with *fdtA* and *fdtB* both from *Aneurinibacillus*
500 *thermoaerophilus* L420-91T, were overexpressed separately to generate dTDP-D-
501 viosamine, dTDP-4-amino-4,6-dideoxy-D-galactose, dTDP-6-deoxy-xylohex-3-ulose,
502 and dTDP-3-amino-3,6-dideoxy-D-galactose, respectively. When a plant
503 glycosyltransferase (arGT3) from *A. thaliana* was introduced into the system,
504 quercetin-4-amino-4,6-dideoxy-D-galactose and quercetin-3-amino-3,6-dideoxy-D-
505 galactose were synthesized (Pandey et al., 2015). Interestingly, when a flexible GT
506 named YjiC from Bacillus licheniformis DSM13 was overexpressed and 3-
507 hydroxyflavone was used as the substrate, the strain could only use dTDP-L-rhamnose
508 as the sugar donor to biosynthesize the corresponding rhamnoside (Pandey et al.,
509 2016b). These studies indicated that GTs from different sources may have their own
510 specificities on both nucleotide sugar donors and acceptors. Therefore, sequence and
511 phylogenetic analysis of different GTs are critical to select corresponding tools to
512 generate desired glycosides.

513 *3.1.11. Quercetin-3-O-N-acetylglucosamine*

514 Another deoxyaminougar, glucosamine or 2-amino-2-deoxy-D-glucose, is the
515 precursor to its *N*-acetylated derivative, *N*-acetylglucosamine. Both glucosamine and
516 *N*-acetylglucosamine can be found in plants, animals, and microbes including bacteria,

yeast, and filamentous fungi (Deng et al., 2005). Saponin glycosaminosides are promising candidates as antifungal and antibacterial drugs. Moreover, the toxicity of saponins can be reduced in the forms of glycosaminosides (Grzywacz et al., 2020). A recent study showed that quercetin-3-*O*-*N*-acetylgalactosamine has the potential to be used as an antioxidant supplement (Xu et al., 2022). Because of these benefits, some researchers have focused on the biosynthesis of quercetin-3-*O*-*N*-acetylglucosamine and its derivatives, including quercetin-3-*O*-*N*-acetylquinovosamine and quercetin-3-*O*-*N*-acetylxylosamine.

Rational engineering of nucleotide sugar metabolic pathways in the hosts can lead to the production of various glycosides through a flexible GT. While the *A. thaliana* enzyme AtUGT78D2 prefers UDP-glucose as a sugar donor, Kim et al. found that AtUGT78D2 could also take UDP-*N*-acetylglucosamine as a substrate *in vitro*. Two *E. coli* mutant strains *E. coli*/Δ*pgm* (Bpgm) and *E. coli*/Δ*galU* (BgalU) were created to reduce the production of UDP-glucose. Due to the relatively higher abundance of UDP-*N*-acetylglucosamine in *E. coli* strains Bpgm and BgalU than the wild type, AtUGT78D2 was able to take UDP-*N*-acetylglucosamine as the sugar donor in the cells. The production titer of quercetin-3-*O*-*N*-acetylglucosamine reached 380.7 mg/L after 24 hours by in strain BgalU, and only 10.4 mg/L of "byproduct" was formed, namely quercetin-3-*O*-glucose which is the main product in wild type *E. coli* (Kim et al., 2012b). This study clearly shows that for flexible GTs, altering the supply of corresponding sugar donors in the host enables the production of desired glycosides.

3.1.12. Quercetin-3-O-N-acetylquinovosamine

To engineer the *E. coli* nucleotide sugar biosynthetic pathway for enhanced supply of target nucleotide sugars, Cho et al. deleted two genes, namely *galU* and *pgm*, from *E. coli*. They then cloned two genes, *Pdeg* (UDP-*N*-acetylglucosamine 4,6-dehydratase) and *Preq* (UDP-4-reductase) from the genomic DNA of *Bacillus cereus* ATCC 14579 for synthesizing UDP-quinovosamine into *E. coli*. Finally, the AtUGT78D2 gene from *A. thaliana* was introduced to catalyze the glycosylation. By comparison, quercetin-3-*O*-*N*-acetylquinovosamine was produced with a higher level using the *galU*-deleted strain. The final production titer of quercetin-3-*O*-*N*-acetylquinovosamine reached 158.3 mg/L (Cho et al., 2016b). This work demonstrates that it is possible to discover unique nucleotide biosynthetic pathways from other hosts, such as *B. cereus* ATCC 14579, and incorporate them into common workhorse strains to create new glycosides.

3.1.13. Quercetin-3-O-N-acetylxylosamine

Another example is the engineered production of quercetin-3-*O*-*N*-acetylxylosamine. To achieve this goal, UDP-*N*-Acetylxylosamine synthase (UXNAcS) from *B. cereus* was introduced into *E. coli* to transform UDP-*N*-acetyl-D-glucosaminuronate into UDP-*N*-acetyl-D-xylosamine. With the help of AtUGT78D2, 160.8 mg/L of quercetin-3-*O*-*N*-acetylxylosamine was produced in the *pgm*-deleted *E. coli* strain (Cho et al., 2016b). In addition, the authors found that increasing the copy number of the expression plasmid is an effective approach for enhancing the product titer. When the cell density reached OD_{600} 8.0, the production reached the maximum level, suggesting that fermentation engineering is an important factor for polishing the production process to maximize system efficacy.

561 *3.1.14. Kaempferol-3-O-glucoside (astragalin)*

562 In addition to quercetin glycosides, kaempferol glycosides also have various
563 bioactivities. Kaempferol-3-*O*-glucoside (astragalin) is a common plant metabolite
564 present in many plants such as *Annona muricata* (Taiwo et al., 2019), *Cressa cretica*
565 (Fawzi et al., 2019), *Chenopodium album* (Mehdi et al., 2018), and *Cuscuta chinensis*
566 (Karna et al., 2019; Tao et al., 2021). Astragalin exhibits antimicrobial activities (Taiwo
567 et al., 2019), antitumor activity (Wang et al., 2021), and many other bioactivities (Riaz
568 et al., 2018). To enable microbial production of this bioactive natural product, Malla et
569 al. deleted three genes, namely *pgi*, *zwf*, and *ushA* in *E. coli* BL21(DE3) strain and
570 overexpressed two genes, namely phosphoglucomutase (*nfa44530*) from *Nocardia*
571 *farcinia* and glucose-1-phosphate uridylyltransferase (*galU*) from *E. coli* K12 that are
572 involved in the synthesis of UDP-glucose from glucose-6-phosphate, to provide
573 sufficient UDP-glucose to produce astragalin from naringenin. Two genes encoding
574 flavanone-3-hydroxylase (*f3h*) and flavonone synthase (*fls1*) were introduced from *A.*
575 *thaliana* into the engineered strain, which convert naringenin into dihydrokaempferol
576 and subsequently kaempferol. Using endogenous UDP-glucose, the GT UGT78K1
577 from *Glycine max* catalyzes the 3-*O*-glucosylation of kaempferol. Glycerol and
578 mannitol were used as the carbon sources for cell growth, leading to the production of
579 astragalin at 109.3 mg/L from 500 mM of naringenin in 60 hours (Malla et al., 2013).
580 Similarly, Pei et al. constructed an engineered *E. coli* strain by introducing favanone-3-
581 hydroxylase (F3H) and favonol synthase (FLS) and obtained kaempferol production at
582 1.18 ± 0.02 g/L from naringenin after optimizing the fed-batch fermentation conditions.

583 Finally, with the employment of AtUGT78D2 and introduction of an efficient UDP-
584 glucose biosynthetic pathway, astragalin was produced at 1.74 ± 0.02 g/L in the
585 resulting strain (Pei et al., 2019).

586 *3.1.15. Kaempferol-3-O-rhamnoside* (*afzelin*)

587 Kaempferol-3-*O*-rhamnoside (afzelin) is known for its antioxidant (Akter et al.,
588 2022a), anti-inflammatory (Zhao et al., 2021), anti-tumor activities (Akter et al., 2022b).
589 Afzelin can also protect against *β*-amyloid-induced cell death, which could serve as a
590 potential treatment of Alzheimer's disease (Yang et al., 2014). Yang et al. biosynthesized
591 the flavonoid glycoside from glucose in *E. coli* instead of feeding substrate such as
592 flavonoids directly into fermentation broth. They initially engineered a tyrosine
593 biosynthetic pathway into *E. coli* to produce tyrosine, the precursor of flavonoids, from
594 glucose. Subsequently, they introduced four flavonoid biosynthetic genes into *E. coli*
595 to synthesize kaempferol from tyrosine, including tyrosine ammonia lyase (TAL), 4-
596 coumaroyl CoA ligase (4CL), chalcone synthase (CHS), and flavonol synthase (FLS).
597 Meanwhile, to increase the supply of tyrosine, four genes including *ppSA*, *tktA*, *aroG*
598 and *tyrA* were overexpressed. Finally, the flavonol-3-*O*-rhamnosyltransferase
599 (*UGT78D1*) that is specific for U(T)DP-rhamnose from *A. thaliana* was introduced,
600 leading to the production of kaempferol-3-*O*-rhamnoside at 57 mg/L after 30 hours
601 (Yang et al., 2014). This approach for direct biosynthesis of flavonoid glycosides from
602 glucose is promising, especially when the aglycons are expensive or not commercially
603 available. In this work, in situ synthesis of the sugar-acceptor kaempferol could be
604 further improved to enhance the production titer of kaempferol-3-*O*-rhamnoside.

605 Therefore, the balance between sugar donor and sugar acceptor is essential for efficient
606 production of flavonoid glycosides. Though most bacteria or fungi cannot naturally
607 synthesize flavonoids, this work demonstrates the possibility of engineering the plant
608 flavonoid biosynthetic pathway into microbes to generate the desired aglycons. This
609 breakthrough makes it feasible to produce valuable flavonoid glycosides using glucose
610 as the sole starting substrate.

611 *3.1.16. Fisetin-3-O-glucoside*

612 Fisetin exhibits antioxidant activity that may promote health conditions (Khan et
613 al., 2013). However, the clinical application of fisetin is limited due to its low water
614 solubility (Lorthongpanich et al., 2022). To deal with this problem, researchers have
615 used enzymatic approaches to prepare fisetin glucosides while preserving its
616 antioxidant activity (Lorthongpanich et al., 2022). The approach of assembling multiple
617 genes in a single vector was applied for engineered production of fisetin-3-*O*-glucoside.
618 Instead of co-expressing various plasmids harboring different genes in the same host
619 strain, a single vector system can decrease the metabolic burden created by different
620 antibiotics supplemented into the fermentation broth. For example, Parajuli et al.
621 assembled *glf*, *glk*, *pgm*, and *galU* into the same vector pIBR181 together with the
622 regiospecific flavonol-3-*O*-glycosyltransferase (UGT78K1) into *E. coli* BL21(DE3).
623 The engineered strain produced 1.178 g of fisetin-3-*O*-glucoside after 48 hours in a 3-
624 L fermentor when 0.9 g of fisetin was supplemented (Parajuli et al., 2015). Assembling
625 various biosynthetic pathway genes into a single vector under distinct promoters
626 presents a promising approach for fine-tuning gene expression. Such an approach can

627 mitigate the metabolic burden resulting from multiple antibiotic selection markers
628 needed in a multi-vector system.

629 *3.1.17. Fisetin-3-O-rhamnoside*

630 Knocking out unnecessary genes in *E. coli* represents an effective and widely used
631 approach for producing appreciable quantities of NDP-sugars. In addition, some
632 researchers proposed the transfer of extracellular glucose into the cells in a more
633 efficient way to increase the pool of UDP-glucose. To achieve this goal, Parajuli et al.
634 introduced the glucose facilitator diffusion protein (*glf*) in the dTDP-L-rhamnose
635 biosynthetic cassette. Specifically, they first introduced the *glf*, *glk*, *pgm*, and *tgs* genes
636 to synthesize dTDP-D-glucose from extracellular glucose. They next completed the
637 dTDP-D-rhamnose biosynthetic system by adding three genes *dh*, *epi*, and *kr* into the
638 same vector pIBR181. Finally, a regiospecific flavonol-3-*O*-rhamnosyltransferase
639 (ArGt-3) gene was cloned separately into pET32(a)+ to generate pET32(a)+/ArGt-3.
640 The dTDP-rhamnose cassette along with pET32(a)+/ArGt-3 were transferred into *E.*
641 *coli* BL21(DE3) for rhamnoside production. As a result, 1.026 g of fisetin-3-*O*-
642 rhamnoside was produced from 0.9 g fisetin after 48 hours of incubation in a 3-L
643 fermentor (Parajuli et al., 2015). Thus, internalizing extracellular glucose from the
644 medium into cells represents a useful method for increasing the intercellular pool of
645 UDP-glucose.

646 *3.1.18. Myricetin-3-O-rhamnoside (myricitrin)*

647 Myricetin-3-*O*-rhamnoside (myricitrin) exhibits higher antioxidant activity than
648 other flavonol rhamnosides (Wu et al., 2008). It also has antinociceptive (Meotti et al.,

649 2006), anti-inflammatory (Shimosaki et al., 2011), hepatoprotective, and antifibrotic
650 activities (Domitrović et al., 2015). To synthesize this valuable natural product in an
651 environmentally-friendly fashion, Thuan et al. constructed *E. coli*
652 BL21(DE3)/Δ*pgi*Δ*zwf* for enhancing the intracellular production of dTDP-*α*-L-
653 rhamnose pool in the cell cytoplasm. Realizing that *E. coli* does not naturally synthesize
654 UDP-rhamnose, the authors focused on using endogenous dTDP-rhamnose. The dTDP-
655 *α*-L-rhamnose gene cassette consisted of two recombinant plasmids, including
656 pTGSDH expressing the *tgs* and *dh* genes, and pAC-EPKR harboring the *epi* and *kr*
657 genes. The GT from *A. thaliana* (*ArGT-3*) was used for rhamnosylation of myricetin,
658 and methylated cyclodextrin was used as a molecular carrier for myricetin. Finally, the
659 maximum titer of *myricetin-3-O-rhamnoside* reached 55.6 μM from 100 μM myricetin
660 under optimized conditions (Thuan et al., 2013b). This work shows that sufficient
661 supply of nucleotide sugars as sugar donors is pivotal for glycoside prouduction in *E.*
662 *coli*.

663 *3.2. Engineered production of flavone glycosides*

664 *3.2.1. Apigenin-7-O-glucoside (apigetrin)*

665 Flavones, such as apigenin, scutellarein, baicalein, and luteolin, possess a core
666 structure that closely resembles that of flavonols, except for the absence of the 3-OH
667 group. While flavones are relatively less abundant than flavonols, they can be readily
668 obtained from fruit peels (Abbas et al., 2017). Apigenin-7-*O*-glucoside (apigetrin) is a
669 potent anti-cancer (Kim et al., 2020; Liu et al., 2020) and anti-inflammatory drug (Wang
670 et al., 2020). Studies have shown that apigetrin offers protection against *Toxoplasma gondii*

(Abugri and Witola, 2020) and can be used to treat anxiety (Kumar and Bhat, 2014), making it an exciting prospect for human health. Unfortunately, this compound is costly and not widely available in plants (Wang et al., 2018). As a result, biosynthetic methods for engineered apigetrin production are being developed to harness its promising health benefits.

Thuan et al. engineered an *E. coli* co-culture system for the *de novo* synthesis of apigetrin. Upstream of the co-culture system were four enzymes that enable the production of apigenin from *p*-coumaric acid, including 4-coumarate: CoA ligase (4CL), chalcone synthase (CHS), chalcone favanone isomerase (CHI), and flavone synthase I (FNSI). The downstream of the co-culture system was constructed to enhance the production of UDP-glucose and express the GT (PaGT3) from *Phytolacca americana* to transform apigenin into apigetrin. By optimizing temperature and media components, the production titer of apigetrin reached 16.6 mg/L, twice that achieved using monoculture (6.7 mg/L) (Thuan et al., 2018a). This study demonstrates that co-culture systems are a viable approach for addressing the limitations of monoculture and improving production by enabling the independent optimization of two engineered pathways, thereby reducing metabolic burden in a single strain.

3.2.2. Scutellarein-7-O-glucoside

Scutellarein-7-*O*-glucuronide, also known as scutellarin, possesses antioxidant, anti-inflammatory, vascular relaxation, anti-platelet, and anti-coagulation properties, as well as myocardial protective effects. It has been employed in the treatment of stroke, myocardial infarction, and diabetic complications (Wang and Ma, 2018). To improve

693 the bioavailability, efficacy, and safety of scutellarein, its glycosides were
694 biosynthesized, thereby expanding the potential applications of this compound. Wang et al.
695 used *S. cerevisiae* as a biocatalyst to produce scutellarein-7-*O*-glucoside. The flavonoid
696 glucosyltransferases (SbGT34) from *Scutellaria baicalensis* Georgi was selected to
697 construct the engineered yeast. By using homologous integration, three glucosidase
698 genes were knocked out, including *SPR1*, *YIR007W,* and *EXG1* that is the key gene to
699 hydrolyze flavonoid glucosides in *S. cerevisiae*. Furthermore, two genes encoding
700 phosphoglucomutase and UTP-glucose-1-phosphate uridylyltransferase were
701 overexpressed in *S. cerevisiae,* which are involved in the biosynthesis of UDP-glucose.
702 The production titer of scutellarein-7-*O*-glucoside reached 1.2 g/L after 54 hours of
703 incubation from 3.5 g scutellare in a 10-L fermentor (Wang et al., 2016). This research
704 suggests that although endogenous glucosidases present in yeast platforms can
705 somewhat impede the biosynthesis of glycosides, deleting the glucosidase genes can
706 still render yeast an appealing host for this purpose.

707 *3.2.3. Baicalein-7-O-glucuronide (baicalin)*

708 Baicalin, also known as baicalein-7-*O*-glucuronide, is a compound extracted from
709 the root of *S. baicalensis* Georgi that exhibits a wide range of biological effects (Gupta
710 et al., 2022; Yang et al., 2016), such as antitumor, antimicrobial, and antioxidant
711 activities (Huang et al., 2019). Moreover, baicalin is used as a reference compound for
712 the quality control of *Scutellaria radix* (Zhao et al., 2016). However, the low
713 bioavailability of baicalin has impeded its clinical use. To address this issue, researchers
714 have attempted to glycosylate baicalein to make more water-soluble derivatives (Kim

et al., 2014).

To produce baicalein in microbes, Yang et al. constructed an engineered *E. coli* BL21(DE3) strain to produce flavonoid glucuronides using a module-based approach. The first module is an endogenous upstream biosynthetic pathway to produce the sugar donor UDP-glucuronic acid. To strengthen the UDP-glucuronic acid synthetic pathway, they modified three genes, namely, *pgm*, *galU* and *ugd* to increase the endogenous level of UDP-glucuronic acid. After identifying Ugd as the rate-determining step in glucuronide production, they introduced this gene into a high-copy plasmid pEG12 under the T7 promoter to maintain the uniform expression. The second module is a heterologous downstream pathway to glucuronidate flavonoids by using a UDP-glucuronosyltransferase (SbUGT) from *S. baicalensis* Georgi. Ultimately, the production yield of baicalin reached 797 mg/L (Yang et al., 2016). This study highlights the significance of identifying and enhancing the rate-limiting step or bottleneck for achieving higher production of glucuronides in engineered E. coli strains, as demonstrated by the upregulation of the *ugd* gene in this research.

3.2.4. Luteolin-7-O-N-acetylglucosaminuronate

Luteolin-7-*O*-*N*-acetylglucosaminuronate was produced using the similar *E. coli* strains for quercetin-3-*O*-*N*-acetylquinovosamine production. To achieve efficient production, Δ*pgm* mutant strain of *E. coli* was used as the starting host. Cho et al. engineered the UDP-*N*-acetylglucosamine 6-dehydrogenase gene (*UDP-GlcNAc 6-DH*) from *B. cereus* NVH 391-98 with codon optimization into *E. coli*. AmUGT10 from *Antirrhinum majus* was then introduced to achieve the production of luteolin-*O*-*N*-

acetylglucosaminuronate at 172.5 mg/L (Cho et al., 2016b). The GT AmUGT10 originally uses UDP-glucuronic acid as a sugar donor and transfers glucuronic acid onto the 7-hydroxy group of luteolin. Interestingly, it also takes UDP-*N*-acetylglucosaminuronate. Moreover, the author found that *E. coli*/Δ*pgm* mutant is more effective (approximately two-fold) than *E. coli*/Δ*galU* mutant for the production of luteolin-7-*O*-*N*-acetylglucosaminuronate. This may be due to a higher level of the precursor UDP-N-acetylglucosamine in the former strain. Hence, it is essential to compare and evaluate different mutant strains during the production optimization process. This study also demonstrated that some GTs are versatile toward different nucleotide sugars, which can be utilized to synthesize various flavonoid glycosides.

3.3. Engineered production of flavanone, flavononol, and anthocyanidin glycosides

3.3.1. Naringenin-7-O-xyloside

Flavanones lack a double bond between C-2 and C-3 and a hydroxyl group at C-3. They are primarily present in citrus fruits and can also be found in aromatic plants. (Leuzzi et al., 2000). Naringenin has many beneficial effects on human health (Venkateswara Rao et al., 2017). Unfortunately, this compound is difficult to absorb after oral administration, limiting its efficacy (Manach and Donovan, 2004). Naringin, a glycosylated form of naringenin, has been found to prevent obesity, heart disease, diabetes, bone disorders, and metabolic syndrome. It also has potential as an anti-inflammatory drug with reduced side effects (Lavrador et al., 2018; Zhao and Liu, 2021). Therefore, there is interest in generating naringenin glycosides to leverage their therapeutic advantages (Joshi et al., 2018; Simkhada et al., 2009a). However, due to the

long synthetic pathway of UDP-D-xylose involving various enzymes, it is relatively difficult to achieve a high production titer of naringenin-7-*O*-xyloside. Simkhada et al. integrated and expressed three genes in *E. coli* BL21(DE3)/Δ*pgi* for the synthesis of UDP-D-xylose, including *galU* from *E. coli* K12, together with *calS8* and *calS9* from *Micromonospora echinospora* spp. *calichensis*. They then introduced the 7-*O*-GT *arGt-4* from *A. thaliana* to yield *E. coli* strain US89Gt-4 to produce naringenin-7-*O*-xyloside (Simkhada et al., 2009a). Further optimization of the culture conditions and gene expression levels could be helpful for enhanced production of naringenin-7-*O*-xyloside.

3.3.2. Taxifolin-3-O-rhamnoside (astilbin)

Flavononols lack a double bond between C-2 and C-3, and there is a hydroxy group at C-3. Taxifolin-3-*O*-rhamnoside (astilbin) is a flavononol glycoside mainly isolated from *Smilax glabra* Roxb (Zhang and Cheung, 2010). Besides its antimicrobial, insecticidal, and antioxidant activities, astilbin also has effects on central nervous system (Alzheimer's disease and Parkinson disease) and cardiovascular system (myocardial ischemia and reperfusion injury and lipolysis) (Sharma et al., 2020). As the main method of acquiring astilbin, plant extraction is still unable to meet the demand of industrial-scale production (Prawat et al., 2012), despite extensive studies on optimizing extraction conditions (Lu et al., 2015). Therefore, researchers have developed novel methods to biosynthesize astilbin via microorganisms.

Thuan et al. used the engineered *E. coli* BL21(DE3)/Δ*pgi*Δ*zwf* to enhance the pool of D-glucose-6-phosphate. To improve the intracellular TDP-L-rhamnose pool, four genes were overexpressed under the control of the strong T7 promoter, including *tgs*

781 from *Thermus caldophilus* GK24, *dh* from *Salmonella thyphimurium* LT2, together
782 with *epi* and *kr* from *Streptomyces antibioticus* Tü99. The engineered strain named *E.*
783 *coli* M3G3 was constructed by introducing an exogenous UDP-glycosyltransferase
784 (*Ar*GT3) from *A. thaliana*. The final production titer of astilbin reached around 15 mg/L
785 from 100 μM taxifolin after 48 hours (Thuan et al., 2017a). This successful example
786 indicates that promoter optimization could facilitate the production of polyphenolic
787 glycosides.

788 *3.3.3. Cyanidin-3-O-glucoside (chrysanthemin)*

789 The glycosylated forms of anthocyanidin are called anthocyanins, which are red,
790 purple, or blue plant water-soluble pigments with anti-oxidative, anti-inflammatory,
791 anticancer, anti-obesity, anti-diabetic, and cardioprotective properties (Yan et al., 2008).
792 One example of an anthocyanin is cyanidin-3-*O*-glucoside, also known as
793 chrysanthemin, which has been found to have gastro-protective and anti-inflammatory
794 properties (Olivas-Aguirre et al., 2016). To biosynthesize this valuable natural product,
795 Yan et al. constructed a metabolic pathway in *E. coli* with four plant genes from
796 different origins, including flavanone 3′-hydroxylase (*F3'H*) from *Malus domestica*,
797 dihydroflavonol-4-reductase (*DFR*) from *Anthurium andraeanum*, anthocyanidin
798 synthase (*ANS*) also from *M. domestica*, and flavonoid-3-*O*-glucosyltransferase (*3-GT*)
799 from *Petunia hybrida*. Through this strain, naringenin was converted into the colored
800 and stable chrysanthemin (Yan et al., 2005). Even though the production titer only
801 reached microgram scale, this work for the first time synthesized plant-specific
802 anthocyanin via microbial fermentation. To increase the production titer of

chrysanthemin, Yan *et. al* manipulated the metabolic network of *E. coli* to enhance the intracellular UDP-glucose pool, which was considered the key metabolic limitation in this study. They also optimized the pH of culture medium and created the fusion proteins of 3GT and ANS. Eventually, the production titer of chrysanthemin was increased to 70.7 mg/L (Yan et al., 2008). The authors noted that anthocyanidins are unstable compounds that could degrade before glycosylation, potentially hindering chrysanthemin production. Therefore, this study highlights the potential of multi-protein complexes to increase precursor concentrations and prevent the degradation of unstable intermediates.

To further increase the production titer of chrysanthemin, Lim et al. successfully achieved a final titer of 350 mg/L through a combination of three approaches: enhancing substrate availability, balancing gene expression, and optimizing cultivation and induction conditions. They first constructed a bicistronic expression cassette to improve the expression of anthocyanidin synthase (ANS) and 3-*O*-glycosyltransferase (3GT). Then, intracellular UDP-glucose was increased by overexpressing another *E. coli* endogenous phosphoglucomutase (*ycjU*) instead of the original *pgm*, which allowed higher production of UDP-glucose than coexpressing Pgm and GalU. More interestingly, they identified several *E. coli* transporter proteins that play an important role in substrate uptake and product secretion, including four efflux pumps (AcrAB, TolC, AaeB, and YadH), one uptake pump (TnaB), as well as one regulator (MarA). Further optimization of culture and induction conditions was also performed (Lim et al., 2015). The work provides a promising approach to develop an inexpensive

825 process for large-scale production of plant-specific anthocyanins from engineered
826 microorganisms, which supports the industrial production of natural food colorants to
827 meet their increased market demand.

828 *3.3.4. Anthocyanidin-3-O-glucoside*

829 Some researchers managed the complete biosynthesis of anthocyanidin-3-*O*-
830 glucosides in *E. coli*. Jones et al. engineered a complex *E. coli* co-culture system to
831 biosynthesize anthocyanidin-3-*O*-glucosides directly from the carbon source. In total
832 fifteen exogenous or modified genes from different sources, including plants and
833 microbes, were distributed into the polyculture system containing four engineered *E.*
834 *coli* strains, including (1) *E. coli* strain rpoA14(DE3) expressing tyrosine ammonia
835 lyase (TAL) produces phenylpropanoic acids from glucose; (2) BL21star^{TM}(DE3)
836 ΔsucCΔfumC with 4-coumarate: CoA ligase (4CL), chalcone synthase (CHS), and
837 chalcone isomerase (CHI) generates flavanones from phenylpropanoic acids; (3)
838 BL21star^{TM}(DE3) harboring flavanone 3'-hydroxylase (F3'H), dihydroflavonol 4-
839 reductase (DFR), and leucoanthocyanidin reductase (LAR) synthesizes flavan-3-ols
840 from flavanones; (4) BL21star^{TM}(DE3) with anthocyanadin synthase (ANS) and 3-*O*-
841 glycosyltransferase (3GT) transforms flavan-3-ols into anthocyanins. By using the
842 polyculture strategy, the authors achieved milligram-per-liter production titer of
843 anthocyanidin-3-*O*-glucosides (Jones et al., 2017). This study is a great example of
844 relieving metabolic burden using a polyculture system, which provides a new method
845 for the rearrangement of complex metabolic pathways in *E. coli* for glycoside
846 production.

3.4. Engineered production of stilbene and curcuminoid glycosides

3.4.1. Resveratrol-3-O-glucoside (piceid), resveratrol-4'-O-glucoside (resveratroloside), and resveratrol glucuronides

Stilbenoids (such as resveratrol) are 1,2-diphenylethene polyphenols that are derivatives of stilbene and have a 14-carbon skeleton with C_6-C_2-C_6 backbone. Specifically, C_2 represents the ethylene bridge that links two differently substituted aromatic rings together. Plants synthesize stilbenoids to protect themselves from pathogens, and they are promising natural products for the development of antimicrobial agents (Mattio et al., 2020). Glycosylation is a prodrug approach that aims to address the low bioavailability issue of resveratrol (Intagliata et al., 2019). Studies have shown that resveratrol-3-*O*-glucoside (piceid) exhibits similar or even improved bioactivities compared to resveratrol. For example, the tyrosinase inhibitory activity of resveratrol-3-*O*-glucoside was reported to be higher than that of resveratrol (Uesugi et al., 2017; Walle, 2011). To achieve the biosynthesis of resveratrol glycosides, Choi et al. first used the resveratrol-producing construct pET-opTLS to synthesize resveratrol from tyrosine, containing the codon-optimized tyrosine ammonia lyase (*tal*) gene from *Saccharothrix espanaensis*, 4-coumarate-CoA ligase (*4cl*) gene from *Streptomyces coelicolor*, and codon-optimized stilbene synthase (*sts*) gene from *Arachis hypogaea*. Next, a UDP-glycosyltransferase gene (*yjiC*) was introduced to create construct pET-opTLYS for synthesizing resveratrol glycosides. Each gene in the system has its own T7 promoter, ribosome-binding site (RBS), and terminator sequence. Finally, recombinant *E. coli* C41(DE3) strain harboring pET-opTLYS produced 2.5 mg/L piceid

and 7.5 mg/L resveratroloside from glucose in modified M9 minimal medium (Choi et al., 2014). Although the final production titers are low, this strategy demonstrates the first *de novo* synthesis of resveratrol glucoside derivatives in *E. coli* from a simple medium.

Thuan et al. used a coculture approach for the synthesis of resveratrol glucosides. They constructed the aglycone-forming pathway and sugar-related pathway in two different cell systems, leading to the *E. coli* RES and *E. coli* RGL strains, respectively. The aglycone biosynthetic pathway contained 4CL and STS to convert *p*-coumaric acid into resveratrol. The UDP-sugar forming and transferring pathway included UDP-glucose pyrophosphorylase (*hasC*) and glucosyltransferase (*PaGT3*) to convert the resveratrol into its glucosides, namely, piceid and resveratroloside. Under the optimized conditions, the production of resveratrol glucosides reached 92 mg /L (236 μM) from 280 μM of *p*-coumaric acid after 60 hours in a 3-L fed batch fermentor (Thuan et al., 2018b). Moreover, engineered production of resveratrol glucuronides was also achieved in *E. coli* recently. Ren et al. identified a new glucuronyltransferase (GcaC) from *Streptomyces chromofuscus* ATCC 49982. After optimizing the pH, temperature, cell density, substrate concentration, and incubation time, around 78 mg/L of resveratrol-4'-*O*-glucuroside and 15 mg/L of resveratrol-3-*O*-glucuroside were produced in engineered *E. coli* BL21(DE3) (Ren et al., 2022a). These studies demonstrate that engineered microbial production is an effective tool for the generation of resveratrol glycosides.

3.4.2. Curcumin glucoside

Curcuminoids, represented by curcumin, are phenolic compounds that are widely utilized as a spice, pigment, food additive, and therapeutic agent. They are the primary constituents in *Curcuma* species and possess a common unsaturated alkyl-linked biphenyl structural feature that accounts for their major pharmacological effects (Amalraj et al., 2017). Curcumin glycosides have diverse and even improved bioactivities. For example, curcumin glucoside has been shown to inhibit α-synuclein oligomer formation, which is relevant to Parkinson's disease (Shrikanth Gadad et al., 2012), while curcumin 4'-*O*-*β*-glucooligosaccharides exhibit anti-allergic activity (Shimoda and Hamada, 2010a). Moreover, curcumin-4'-*O*-*β*-glucoside and curcumin-4'-*O*-*β*-2-deoxyglucoside have been found to possess enhanced anticancer activities compared to curcumin (Gurung et al., 2017). Additionally, the antioxidant property of curcumin-*β*-diglucoside is stronger than curcumin, and it also exhibits higher antibacterial properties against *Staphylococcus aureus* and *E. coli* than curcumin (Parvathy et al., 2009). Notably, Singh et al. reported the first curcumin glucoside biosynthesis in *Atropa belladonna* hairy roots. By heterologous expression of key curcumin biosynthetic pathway genes such as Diketide-CoA synthase (DCS) and curcumin synthase (CURS3) from *Curcuma amada*, together with a glucosyltransferase gene (CaUGT2) from *Catharanthus roseus* in *A. belladonna*, the highest content of curcumin monoglucoside reached 32.63 ± 2.27 μg/g DW in shaker flasks (Singh et al., 2021). Therefore, *A. belladonna* hairy roots provide an option for the production of high-value polyphenolic glycosides in the future.

3.5. Engineered production of other phenolic glycosides

913 *3.5.1. Vanillin-4-O-glucoside*

914 Vanillin is a significant flavoring agent that was initially isolated from Vanilla
915 planifolia. It has a global market value of 180 million US dollars, with an annual
916 worldwide demand of approximately 16,000 tons (Pandey et al., 2018). Vanillin also
917 possesses antioxidant, antifungal and antidepressant activities (Fitzgerald et al., 2005;
918 Shoeb et al., 2013; Tai et al., 2011). Interestingly, vanillin glycosides are also naturally
919 present in the producer organism *Vanilla planifolia* (Ramachandra Rao and Ravishankar,
920 2000). Rather than relying on common chemical synthesis to produce vanillin from
921 fossil hydrocarbons and lignin (Pandey et al., 2018), a more sustainable approach such
922 as microbial production of vanillin is desirable.

923 Brochado et al. constructed a *de novo* biosynthetic pathway in the cell factory of
924 *S. cerevisiae* for improved production of vanillin glycosides from glucose. To convert
925 3-dehrydroshikimate into vanillin, four genes were introduced, including 3-
926 dehydroshikimate dehydratase (*3DSD*), aryl carboxylic acid reductase (*ACAR*),
927 phosphopantetheine transferase (*PPTase*), and *O*-methyltransferase (*hsOMT*) from
928 *Podospora pausiceta*, *Nocardia sp.*, *Escherichia coli* and *Homo sapiens*, respectively.
929 A plant family 1 GT from *Arabidopsis thaliana* (UGT72E2) was next introduced for
930 biosynthesizing vanillin-4-*O*-glucoside. By using an *in silico* algorithmic method,
931 namely, minimization of metabolic adjustment (MOMA) as biological objective
932 function, the *S. cerevisiae* genome-scale stoichiometric model was applied to identify
933 and select target reactions via OptGene. The final production titer of vanillin-4-*O*-
934 glucoside reached 500 mg/L (Brochado et al., 2010). This study demonstrates the

applicability of *in silico* modelling tools for overproduction of a product from a multistep heterologous pathway in a eukaryotic system. It suggests that the genetic background of a cell factory is important for efficient production of a given product. Hansen et al. used a similar method to achieve the *de novo* biosynthesis of vanillin from glucose in fission yeast *Schizosaccharomyces pombe*, with the exception of deleting the alcohol dehydrogenase gene (*ADH6*) to prevent the reduction of vanillin to vanillyl alcohol. Their efforts led to a final production titer of 119 mg/L of vanillin-4-*O*-glucoside (Hansen et al., 2009). These studies collectively demonstrate the potential of yeasts as organisms for the production of vanillin and its glucosides.

3.5.2. Tyrosol-8-O-glucoside (salidroside)

Salidroside is the glucoside of tyrosol and is considered the primary bioactive compound found in Tibetan Ginseng *Rhodiola* (Xu et al., 1998). It has been shown to have significant adaptogenic effects, including the treatment of anoxia, microwave radiation, and fatigue, as well as the ability to slow down the aging process (Gen-Xiang et al., 2010; Li, M. et al., 2008) Additionally, it can prevent cardiovascular diseases and cancer (Xie et al., 2020; Zhao, C.C. et al., 2021). However, the conventional extraction method currently employed for obtaining salidroside cannot keep up with the growing demand due to the slow growth of wild *Rhodiola* and the limited amount of salidroside that can be produced (Stepanova et al., 2021). To address these challenges, Xue et al. developed a new method for salidroside production by expressing the GT from *Rhodiola sachalinensis* UGT72B14 in *E. coli*. In order to optimize the expression of UGT72B14 in *E. coli* without altering the amino acid sequence, they performed codon

optimization of this gene, which involved changing a total of 278 nucleotides and decreasing the G+C content to 51.05%. As a result, the final salidroside production reached 6.7 mg/L in both batch and fed-batch cultivation, which was 3.2 times higher than the production levels achieved using the wild-type UGT72B14 (Xue et al., 2016). This study demonstrate that the codon-optimized approach can tackle the issue of poor expression of plant UGTs in microorganisms for glycoside production.

Bai et al. first used the pyruvate decarboxylase (*ARO10*) and endogenous alcohol dehydrogenases (*ADH*) to convert 4-hydroxyphenylpyruvate into tyrosol in *E. coli*. Then, by overexpressing the L-tyrosine biosynthetic genes in *E. coli* and eliminating competing pathway genes such as *tyrR*, *pykA*, *pykF*, and *pheA*, the metabolic flux towards the intermediate 4-hydroxyphenylpyruvate was enhanced and tyrosol production was improved. Finally, the GT UGT73B6 from *R. sachalinensis* was introduced into the recombinant strain, yielding 56.9 mg/L of salidroside (Bai et al., 2014). This study represents the first unique artificial biosynthetic pathway in *E. coli* for the production of salidroside from glucose. Sun et al. designed a syntrophic *E. coli* coculture system to produce salidroside. The coculture system included the aglycone strain for biosynthesis of tyrosol and the glycoside strain for production of salidroside. The aglycone strain harbored the decarboxylase gene (*synkdc4*) from *Pichia pastoris* GS115 for biosynthesis of tyrosol and the glycoside strain contained the GT gene (*synugt85a1*) from *A. thaliana* for the biosynthesis of salidroside. Through the syntrophic coculture approach, salidroside was produced at 6.03 g/L after balancing the metabolic pathway strength (Liu et al., 2018). This study represents the first instance of

de novo production of salidroside using the *E. coli* coculture system, and it holds potential for the production of other essential natural product glycosides.

3.5.3. Hydroquinone glucoside (arbutin)

Arbutin exhibits a strong inhibitory effect on tyrosinase activity, which supports its use as a skin depigmenting agent (Boo, 2021; Draelos et al., 2020; Hori et al., 2004). Moreover, arbutin has anti-inflammatory (Lee and Kim, 2012), antibacterial (Ma et al., 2019), and antitumor properties (Li et al., 2011). It is also effective in treating urinary tract infections, kidney stones, and cystitis (Schindler et al., 2002), along with asthma and coughs (Wang et al., 1994). Engineered production of arbutin has been achieved in microbes. Arend et al. characterized a novel glucosyltransferase (AS) with relatively low substrate specificity from plant cell suspension cultures of *Rauvolfia serpentina*, and it was expressed in *E. coli* with high plant-specific glucosylation efficiencies, resulting in a yield of 250 mg/L arbutin after 36 hours of growth (Arend et al., 2001). Shang et al. modified the biosynthetic pathways in *Yarrowia lipolytica* to produce arbutin. They codon-optimized three genes and heterologously expressed them in *Y. lipolytica*, including chorismate pyruvate-lyase (*UbiC*), 4-hydroxybenzoate 1-hydroxylase (*MNX1*), and hydroquinone glucosyltransferase (*AS*). Furthermore, seven arbutin-biosynthetic genes were overexpressed to maximize arbutin production, and the maximum arbutin titer of 8.6 ± 0.7 g/L was achieved in the final engineered strain po1f-At09 (Shang et al., 2020). This research shows the potential of the shikimate pathway in *Y. lipolytica* for the production of hydroquinone glycosides from glucose.

1000 **Table 1.** Engineered production of various polyphenolic glycosides.

Glycosides	GT	GT origin	Sugar donor	Sugar acceptor or starting material	Host	Genetic modification (Knockout or overexpression)	Titer (mg/L)	Method	Ref.
Flavonols---Quercetin, Kaempferol, Fisetin, Myricitrin									
Quercetin-3-*O*-glucoside (Isoquercetin)	UGT73B3	*A. thaliana*	UDP-Glu	Quercetin	*E. coli*	*Δpgi*	3, 900	1, 4	(Xia and Eiteman, 2017)
	BbGT	*B. bassiana*	UDP-Glu	Quercetin	*S. cerevisiae*	/	99	1	(Ren et al., 2022c)
Quercetin-3-*O*-glucuronide (Miquelianin)	VvUGT	*V. vinifera*	UDP-GluA	Quercetin	*E. coli*	*ΔaraA / ugd* ↑ (*E. coli*)	687	1, 4, 5	(Kim et al., 2015)
	VvGT5	*V. vinifera*	UDP-GluA	Quercetin	*E. coli*	glk ↑ (*Z. mobilisi*) / *pgm2* ↑ (*B.licheniformis*) / *galU* ↑ (*E. coli*) / *ugd* ↑ (*E. coli*)	30	1, 5	(Pandey et al., 2019)
Quercetin-3-*O*-galactoside (Hyperoside)	F3GT	*P. hybrida*	UDP-Gal	Quercetin	*E. coli*	*Δpgi / Δagp / ΔushA / ΔgalETKM / ugpA* ↑ (*B. bifidum*) / *galE* ↑ (*E. coli*)	940	1, 4, 5	(De Bruyn et al., 2015c)
	PhUGT	*P. hybrida*	UDP-Gal	Quercetin	*E. coli*	*UGE* ↑ (*O. sativa*)	280	1, 5	(Kim et al., 2015)
Quercetin-3-*O*-xyloside	ArGT-3	*A. thaliana*	UDP-Xyl	Quercetin	*E. coli*	*Δpgi / Δzwf / ΔushA / nfa44530* ↑ (*N.*	23.78	1, 4, 5	(Pandey et al., 2013)

						farcinica) / *galU* ↑ (*E. coli*) / *calS8* ↑ and *calS9* ↑ (*M. echinospora*)			
	AtUGT78D3	*A. thaliana*	UDP-Xyl	Quercetin	*E. coli*	*ΔarnA* / *UXS* ↑ (*A. thaliana*) / *ugd* ↑ (*E. coli*)	160	1, 4, 5	(Han et al., 2014)
Quercetin-3-*O*-rhamnoside (Quercitrin)	ArGT-3	*A. thaliana*	UDP-Rha	Quercetin	*E. coli*	*Δpgi*/ *tgs* ↑ (*T. caldophilus*) / *dh* ↑ (*S. thyphimurium*) / *epi* ↑ and *kr* ↑ (*S. antibioticus*)	24	1, 4, 5	(Simkhada et al., 2010)
	AtUGT78D1	*A. thaliana*	UDP-Rha	Quercetin	*E. coli*	*ΔrfbD* / *rhm* ↑ (*A. thaliana*)	150	1, 4, 5	(Kim et al., 2012a)
	RhaGT	*A. thaliana*	UDP-Rha	Quercetin	*E. coli*	*Δpgi* / *Δagp* / *ΔushA* / *ΔgalETKM* / *ugpA* ↑ (*B. bifidum*) / *MUM4* ↑ (*A. thaliana*)	1,120	1, 4, 5	(De Bruyn et al., 2015c)
Quercetin-3,7-*O*-bisrhamnoside	AtUGT78D1 (3-OH) AtUGT89C1(7-OH)	*A. thaliana*	UDP-Rha	Quercetin	*E. coli*	*RHM2* ↑ (*A. thaliana*)	67.4	1, 5	(Kim et al., 2013)
Quercetin-3-O-glucoside-7-*O*-rhamnoside	AtUGT78D2 (3-OH) AtUGT89C1 (7-OH)	*A. thaliana*	UDP-Glu and UDP-Rha	Quercetin	*E. coli*	/	67	1	(Kim et al., 2013)
Quercetin-3-*O*-alloside	ArGT-3	*A. thaliana*	UDP-All	Quercetin	*E. coli*	*Δpgi* / *tgs* ↑ (*T. caldophilus*) / *dh* ↑ (*S.*	NA (able	1, 4, 5	(Simkhada et al.,

						thyphimurium) / *GerFK* ↑ (*Streptomyces* sp.)	to isolate)		2010)
Quercetin-3-*O*-taloside	AtUGT78D1	*A. thaliana*	UDP-Tal	Quercetin	*E. coli*	*ΔgalU* / *ΔrffA* / *ΔrfbD* / *tll* ↑ (*A. actinomycetemcomitans*)	98	1, 4, 5	(Yoon et al., 2012)
Quercetin-3-*O*-4-deoxy-4-formamido-L-arabinoside	AtUGT78D3	*A. thaliana*	UDP-Ara4FN	Quercetin	*E. coli*	*ugd* ↑ / *arnA* ↑ / *arnB* ↑	70	1, 5	(Kim et al., 2010)
Quercetin deoxyaminosides	ArGT-3	*A. thaliana*	dTDP-deoxyaminosugars	Quercetin	*E. coli*	*Δpgi* / *Δzwf* / *ΔgalU* *tgs* ↑ (*T. caldophilus* GK2) / *dh* ↑ (*S. typhimurium* LT2) *gerB* ↑ / (*Streptomyces* sp. GERI-155) / *wecE* ↑ (*E. coli* K-12) / *fdtA* ↑ and *fdtB* ↑ (*A. thermoaerophilus* L420-91T)	NA	1, 4, 5	(Pandey et al., 2015)
Quercetin-3-*O*-*N*-acetylglucosamine	AtUGT78D2	*A. thaliana*	UDP-GluNAc	Quercetin	*E. coli*	*Δpgm* / *ΔgalU*	380.7	1, 4	(Kim et al., 2012b)
Quercetin-3-*O*-*N*-acetylquinovosamine	AtUGT78D2	*A. thaliana*	UDP-quinovosamine	Quercetin	*E. coli*	*Δpgm* / *ΔgalU* / *Pdeg* ↑ and *Preq* ↑ (*B. cereus* ATCC 14579)	158.3	1, 4, 5	(Cho et al., 2016b)
Quercetin-3-*O*-*N*-	AtUGT78D2	*A. thaliana*	UDP-*N*-acetyl-D-	Quercetin	*E. coli*	*Δpgm* / UXNAcS ↑ (*B.*	160.8	1, 4, 5	(Cho et

acetylxylosamine			glucosaminuronate			*cereus* ATCC 14579)			al., 2016b)
Kaempferol-3-*O*-glucoside (Astragalin)	UGT78K1	*G. max*	UDP-Glu	Naringenin	*E. coli*	*Δpgi / Δzwf / ΔushA / nfa44530* ↑ (*N. farcinia*) / *galU* ↑ (*E. coli* K12) / *f3h* ↑ / *fls1* ↑ (*A. thaliana*)	109.3	1, 2, 4, 5	(Malla et al., 2013)
	AtUGT78D2	*A. thaliana*	UDP-Glu	Naringenin	*E. coli*	*f3h* ↑ (*C. sinensis*) / *fls1* ↑ (*C. unshiu*)	1738.5	1, 2	(Pei et al., 2019)
Kaempferol-3-*O*-rhamnoside (Afzelin)	UGT78D1	*A. thaliana*	UDP-Rha	Glucose	*E. coli*	*TAL* ↑ (*S. espanaensis* ATCC 51144) / *4CL* ↑ (*Oryza sativa*) / FLS ↑ and *CHS* ↑ (*P. euramericana*) / *ppSA* ↑, *tktA* ↑, *aroG* ↑, and *tyrA* ↑ (*E. coli* K12)	57	1, 2	(Yang et al., 2014)
Fisetin-3-*O*-glucoside	UGT78K1	*G. max*	UDP-Glu	Fisetin	*E. coli*	*Glf* ↑ and *glk* ↑ (*Z. mobilis*) / *pgm* ↑ (*B. licheniformis* DSM 13) / and *galU* ↑ (*E. coli* K-12)	1178	1, 5	(Parajuli et al., 2015)
Fisetin-3-*O*-rhamnoside	ArGT-3	*A. thaliana*	dTDP-Rha	Fisetin	*E. coli*	*Glf* ↑ and *glk* ↑ (*Z. mobilis*) / *pgm* ↑ (*B. licheniformis* DSM 13) / and *tgs* ↑ (*E. coli* K-12) / *dh* ↑ (*S.*	1026	1, 5	(Parajuli et al., 2015)

						enterica LT2) / *epi* ↑ and *kr* ↑ (*S. antibioticus* Tu99)			
Myricetin-3-*O*-rhamnoside (Myricitrin)	ArGT-3	*A. thaliana*	dTDP-Rha	Myricetin	*E. coli*	*Δpgi / Δzwf / tgs* ↑ / *dh* ↑ / *epi* ↑ / *kr* ↑ (NA)	25.8	1, 4, 5	(Thuan et al., 2013a)
Flavones-Apigenin, Scutellarein, Baicalein, Luteolin									
Apigenin-7-*O*-glucoside (Apigetrin)	PaGT3	*P. americana*	UDP-Glu	*p*-coumaric acid	*E. coli*	4CL-2 ↑ (*N. tabacum*) / CHS ↑ and CHI ↑ (*P. hybrida*) ↑ / FNSI (*P. crispum*)	16.6	1, 3	(Thuan et al., 2018a)
Scutellarein-7-*O*-glucoside	SbGT34	*S. baicalensis*	UDP-Glu	Scutellare	*S. cerevisiae*	*ΔEXG1 / ΔSPR1 / ΔYIR007W / PGM2* ↑ and *UGP1*↑ (*S. cerevisiae*)	1,200	1, 4, 5	(Wang et al., 2016)
Baicalein-7-*O*-glucuronide (Baicalin)	SbUGT	*S. baicalensis*	UDP-GluA	Baicalein	*E. coli*	*pgm* ↑, *galU* ↑ and *ugd* ↑ (*E. coli*)	797	1, 5	(Yang et al., 2016)
Luteolin-7-*O*-*N*-acetylglucosaminuronate	AmUGT10	*A. majus*	UDP-*N*-acetylglucosaminuronate	Luteolin	*E. coli*	*Δpgm / UDP-GlcNAc 6-DH* ↑ (*B. cereus*)	172.5	1, 4, 5	(Cho et al., 2016a)
Flavanones, flavononols and anthocyanidins -Naringenin, Taxifolin, Cyanidin									
Naringenin-7-*O*-xyloside	ArGT-4	*A. thaliana*	UDP-Xyl	Naringenin	*E. coli*	*Δpgi / galU* ↑ (*E. coli*) / *calS8* ↑ and *calS9* ↑ (*M. echinospora*)	NA	1, 4, 5	(Simkhada et al., 2009b)
Taxifolin-3-*O*-rhamnoside (Astilbin)	ArGT-3	*A. thaliana*	dTDP-Rha	Taxifolin	*E. coli*	*Δpgi / Δzwf tgs* ↑ (*T. caldophilus*) /	15	1, 4, 5	(Thuan et al., 2017b)

						dh ↑ (*S. typhimurium*) / *epi* ↑ and *kr* ↑ (*S. antibioticus*)			
Cyanidin-3-*O*-glucoside (Chrysanthemin)	3GT	*P. hybrida*	UDP-Glu	Naringenin	*E. coli*	*Pgm* ↑ and *galU* ↑ (*E. coli*) / *F3'H* ↑ (*M. domestica*) / *DFR* ↑ (*A. andraeanum*) / *ANS* ↑ (*M. domestica*)	70.7	1, 2, 5	(Yan et al., 2005; Yan et al., 2008)
	3GT	*A. thaliana*	UDP-Glu	Catechin	*E. coli*	*ycjU* ↑ (*E. coli*) / *AcrAB* ↑, *TolC* ↑, *AaeB* ↑, *YadH* ↑, *TnaB* ↑, and *MarA* ↑ (*E. coli*) / *ANS* ↑ (*P. hybrida*)	350	1, 2	(Lim et al., 2015)
Anthocyanidin-3-*O*-glucoside	3GT	*A. thaliana*	UDP-Glu	Glucose	*E. coli*	*TAL* ↑ / *4CL* ↑ / *CHS* ↑ / *CHI* ↑ / *F3'H* ↑ / *DFR* ↑ / *LAR* ↑ / *ANS* ↑	NA	1, 2, 3	(Jones et al., 2017)
Stilbenes and curcuminoids-Resveratrol, Curcumin									
Resveratrol-3-*O*-glucoside (Piceid/ Polydatin)	YjiC	*B. licheniformis*	UDP-Glu	Tyrosine	*E. coli*	*tal* ↑ (*S. espanaensis*) / *4cl* ↑ (*S. coelicolor*) / *sts* ↑ (*A. hypogaea*)	2.5	1, 2	(Choi et al., 2014)
Resveratrol-4'-*O*-glucoside (Resveratroloside)	YjiC	*B. licheniformis*	UDP-Glu	Tyrosine	*E. coli*	*tal* ↑ (*S. espanaensis*) / *4cl* ↑ (*S. coelicolor*) / *sts* ↑ (*A. hypogaea*)	7.5	1, 2	(Choi et al., 2014)
Resveratrol-glucosides (Piceid/Polydatin and Resveratroloside)	*PaGT3*	*P. americana*	UDP-Glu	*p*-coumaric acid	*E. coli*	*Δ*pgi / *Δ*zwf / *4CL* ↑ (*N. tabacum*) / *STS* ↑ (*V. vinefera*) / *hasC* ↑ (*S.*	92	1, 2, 3, 4, 5	(Thuan et al., 2018c)

						zooepidemicus)			
Resveratrol-3-*O*-glucuronide	GcaC	*S. chromofuscus*	UDP-GluA	Resveratrol	*E. coli*	/	15	1	(Ren et al., 2022a)
Resveratrol-4'-*O*-glucuronide	GcaC	*S. chromofuscus*	UDP-GluA	Resveratrol	*E. coli*	/	78	1	(Ren et al., 2022a)
Curcumin-glucoside	CaUGT2	*C. roseus*	UDP-Glu	Feruloyl-CoA	*A. belladonna*	DCS ↑ and CURS3 ↑ (*C. amada*)	32.6	1, 2	(Singh et al., 2021)
Other phenolic glycosides-Vanillin, Tyrosol, Hydroquinone									
Vanillin-4-*O*-glucoside	UGT72E2	*A. thaliana*	UDP-Glu	Glucose	*S. cerevisiae*	*3DSD* ↑ (*P. pausiceta*) / *ACAR* ↑ (*Nocardia sp.*) / *PPTase* ↑ (*E. coli*) / *hsOMT* ↑ (*H. sapiens*)	500	1, 2	(Brochado et al., 2010)
	UGT72E2	*A. thaliana*	UDP-Glu	Glucose	*S. pombe*	*ΔADH6* / *3DSD* ↑ (*P. pausiceta*) / *ACAR* ↑ (*Nocardia sp.*) / *PPTase* ↑ (*C. glutamicum*) / *hsOMT* ↑ (*H. sapiens*)	119	1, 2	(Hansen et al., 2009)
Tyrosol-8-O-glucoside (salidroside)	UGT72B14	*R. sachalinensis*	UDP-Glu	Tyrosol	*E. coli*	/	6.7	1	(Xue et al., 2016)
	UGT73B6	*R. sachalinensis*	UDP-Glu	4-hydroxyphenylpyruvate	*E. coli*	*ΔtyrR* / *ΔpykA* / *ΔpykF* / *ΔpheA* / *ARO10* ↑ (*S. cerevisiae*) / *ADH* ↑ (*E. coli*)	56.9	1, 2, 4	(Bai et al., 2014)

	UGT85A1	*A. thaliana*	UDP-Glu	Xylose	*E. coli*	*ΔushA* / *pgm* ↑ / *galU* ↑ / *kdc4* ↑ (*P. pastoris*)	6,030	1, 2, 3, 4, 5	(Liu et al., 2018)
Hydroquinone glucoside (Arbutin)	AS	*R. serpentina*	UDP-Glu	Hydroquinone	*E. coli*	/	250	1	(Arend et al., 2001)
	AS	*R. serpentina*	UDP-Glu	Glucose	*Y. lipolytica*	*UbiC* ↑ *(E. coli)* / *MNX1* ↑ (*C. parapsilosis*)	8,600	1, 2	(Shang et al., 2020)

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3.6. *Biosynthetic approaches for the production of bioactive polyphenolic O-glycosides*

Although wild type strains are frequently used to generate novel polyphenolic glycosides (Ren et al., 2022b), achieving efficient production can be challenging due to limited NDP-sugars and low expression levels of endogenous glycosyltransferase in these strains (Pandey et al., 2018). To overcome these challenges, five main strategies have been employed to engineer the metabolic pathway in microorganisms for biosynthesizing bioactive polyphenolic glycosides (Fig. 4a). Each method presents opportunities and challenges related to NDP-sugar supply, metabolic burden, expression levels, and cell growth. In this review, we summarize the advantages, challenges, and efforts associated with each approach, providing insights for researchers seeking to produce health-beneficial polyphenolic glycosides (Fig. 4b).

To begin with, introducing a heterologous GT into microbes (Method 1) is commonly used approach to biosynthesize polyphenolic glycosides. Using a microbial strain overexpressing a GT, the engineered production of polyphenolic glycosides is easier to achieve due to the elevated enzyme levels. For instance, quercetin glucosides and resveratrol glucuronides have been successfully produced by overexpressing a glucosyltransferase and a glucuronyltransferase from *B. bassiana* ATCC 7159 and *S. chromofuscus* ATCC 49982 *in E. coli*, respectively (Ren et al., 2022a; Ren et al., 2022c).

However, some exogenous polyphenolic compounds are costly, which hinders the industrial production of their corresponding glycosides. To solve this problem, researchers successfully performed the *de novo* or semi-biosynthesis of polyphenolic glycosides by co-expressing the aglycon biosynthetic gene cassette together with the

dedicated GT in microorganisms (Method 2). An example is the engineered production of kaempferol-3-*O*-rhamnoside from glucose by overexpressing a kaempferol biosynthetic gene cassette and a rhamnosyltransferase from *A. thaliana* in *E. coli* (Yang et al., 2014). This approach offers the advantage of reducing costs for expensive substrates, but it also poses a disadvantage of increasing metabolic burden to the cells. The use of multi-plasmid systems may further exacerbate this burden, as it requires different antibiotics for fermentation to select the correct transformants, and the expression of multiple enzymes demands large amounts of building blocks, reducing power, and ATP. As an alternative, all genes can be cloned into a single vector to minimize the use of antibiotics, but the cells still need to manage the additional metabolic burden (Parajuli et al., 2015).

Complex biosynthetic engineering and the expression of several heterologous genes can impose a significant burden on host cells, leading to low production titers of target compounds. To mitigate the burden caused by multiple enzyme expressions in a single engineered strain, synthetic microbial co-culture or polyculture techniques are emerging strategies for producing polyphenolic glycosides (Method 3). Co-culture or polyculture systems can distribute various biosynthetic genes into different strains. Therefore, these individually manipulated strains can work together to balance the building blocks and many other cofactors, thus achieving the effective production of glycosides. It is a general approach to use a strain or multiple strains to biosynthesize the sugar acceptor substrate that can be excreted to the culture medium, which is then taken up by another engineered strain that contains the nucleotide sugar (intercellular)

biosynthetic enzymes and the dedicated GT. Examples include the coculture system for the production of apigenin-7-*O*-glucoside (Thuan et al., 2018a) and the polyculture system for the production of anthocyanidin-3-*O*-glucoside (Jones et al., 2017).

To further increase the production titer of polyphenolic glycosides, researchers have attempted to increase the pool of NDP-sugars. Flux rewiring of the sugar biosynthetic pathway by deletion of genes in the competing pathways (Method 4) or overexpressing endogenous and exogenous NDP-sugar biosynthetic genes in the host (Method 5) are two common ways to increase the NDP-sugar supply. Representative examples are the engineered production of quercetin-3-*O*-glucoside (Xia and Eiteman, 2017) and fisetin-3-*O*-glucoside (Parajuli et al., 2015), respectively. In Method 5, various non-natural NDP-sugars can also be generated in the engineered strain for producing unnatural flavonoid glycosides (Pandey et al., 2015). In some cases, codon optimization of the introduced genes and upregulation of the copy number of the expression plasmid are necessary for the efficient production of NDP-sugars (Cho et al., 2016b).

All of the methods described above ultimately undergo scale-up using a bioreactor or enlarged flask fermentation to achieve engineered production of target glycosides (Fig. 4a). Fermentation engineering is a critical step to improve production titer, which includes optimizing culture media, carbon and nitrogen sources, cultivation time, molar concentration of substrates, agitation, dissolved oxygen, pH, temperature, and many other factors. As the platform strain for producing diverse polyphenolic glycosides, *E. coli* has a well exploited central carbon metabolic pathway and can produce various

nucleotide sugars. Moreover, by introducing non-natural nucleotide sugar biosynthetic pathways into *E. coli*, an increasing number of unnatural polyphenolic glycosides have been synthesized to provide bioactive candidates for drug discovery. Therefore, we summarize the biosynthetic pathways of common NDP-sugars in the following section. In addition, enzyme engineering or direct evolution of GTs to increase the production titer or expand the chemical reservoir of polyphenolic *O*-glycosides will also be discussed.

Fig. 4

4. Biosynthetic pathways of diverse nucleotide sugar donors and enzyme engineering of GTs for the production of various polyphenolic glycosides

4.1. Biosynthetic pathways of diverse nucleotide sugar donors

Nucleotide sugars commonly serve as sugar donors for glycosylation. The phosphonucleotidyl moiety in the nucleotide sugars not only works as the leaving group during glycosylation but also functions as the recognition target for GTs. Although nucleotide monophosphate (NMP) sugars such as cytidine monophosphate (CMP) sugar can be used as an activated monosaccharide, nucleotide diphosphate (NDP) sugar is the most well-researched activated monosaccharide that can be used by GTs in the cells, including uridine diphosphate (UDP) sugar, deoxythymidine diphosphate (TDP or dTDP) sugar, adenosine diphosphate (ADP) sugar, cytidine diphosphate (CDP) sugar, and guanosine diphosphate (GDP) sugar. Among them, UDP sugars are the most common sugar donors (Thibodeaux et al., 2008).

The diversity of nucleotide sugars is limited in plants because they only contain

common nucleotide sugars, such as UDP-glucose, UDP-glucuronic acid, UDP-rhamnose, UDP-xylose, and UDP-arabinose (Bowles et al., 2006). Therefore, it is of great interest to expand the pool of NDP-sugars, including generating unnatural sugar donors, to create structural diversity in polyphenolic glycosides products (Blanchard and Thorson, 2006; Thibodeaux et al., 2007; Thibodeaux et al., 2008; Zheng et al., 2022). On the other hand, insufficient formation of NDP-sugars can result in low production titer of desired glycosides. Hence, engineering strategies have been applied to introduce active NDP-sugar formation routes into microbial hosts.

Currently, there are three main pathways to generate common natural nucleotide sugar donors, namely, the synthase pathway, phosphorylase pathway, and kinase pathway (Fig. 5). (1) the synthase pathway directly forms nucleotide sugar from a disaccharide. For example, UDP-glucose can be condensed from UDP and sucrose under the catalysis of sucrose synthase (SUS). UDP is biosynthesized from 6-phosphogluconolactone through the pentose phosphate pathway, and 6-phosphogluconolactone is generated from glucose 6-phosphate (G-6-P) via G-6-P dehydrogenase (Zwf) (Fig. 6). Another example is trehalose synthase, which can generate UDP-glucose directly from trehalose (Masada et al., 2007; Ryu et al., 2011); (2) the phosphorylase pathway uses inorganic phosphate to cleave disaccharides for producing activated sugar 1-phosphate without the consumption of ATP. Then, sugar 1-phosphate can be subsequently coupled with a nucleotidylyltransferase to yield the corresponding NDP-sugar (Desmet and Soetaert, 2012). Glucose 1-phosphate (G-1-P) and its corresponding monosaccharide can be formed by sucrose phosphorylase

(Sprogøe et al., 2004), cellobiose phosphorylase (de Groeve et al., 2011), or maltodextrin phosphorylase (Nahálka, 2008). Specifically, glucose and G-1-P can be generated from cellobiose via cellobiose phosphorylase (CBP). Similarly, fructose and G-1-P can be formed from sucrose via sucrose phosphorylase (BaSP) (Fig. 6). (3) The kinase pathway uses both kinase and nucleotidylyltransferase to form NDP-sugars. Kinases are normally ATP-dependent and use monosaccharides to generate sugar 1-phosphate. Phosphosugar mutases are often needed to convert sugar 6-phosphate into sugar 1-phosphate.

Each of these three pathways provides advantages and drawbacks with respect to the generation of NDP-sugars. UDP is considered to be a product inhibitor of UDP-glucosyltransferase (Masada et al., 2007; Michlmayr et al., 2015). All three pathways can recycle the UDP released from the glycosylation process. The difference is that the synthase pathway can recycle UDP directly to generate UDP-glucose, which can further increase the glycosylation reaction rate (Masada et al., 2007). However, equilibrium constants of many synthases, such as cellulose synthase or lactose synthase, are unfavorable, which restricted the synthesis of NDP-sugars (Field, 2011). Low affinity of these synthases to corresponding disaccharide further impede the generation of NDP-sugar. For example, the K_m value (for trehalose) of the trehalose synthase from *Pyrococcus horikoshii* is 25 mM (Ryu et al., 2011). Similarly, the K_m value (for sucrose) of the sucrose synthase from *Solanum tuberosum* L. is 92 mM (Römer et al., 2004). Compared to sucrose synthase, sucrose phosphorylase has much higher affinity to sucrose with a K_m value of around 1 mM (Aerts et al., 2011), making it more efficient

to produce NDP-sugars. Monosaccharides such as fructose produced from sucrose by sucrose phosphorylase can make the strains more tolerable to acidic conditions and osmotic stress (De Bruyn et al., 2015a). The synthase and phosphorylase routes can generate monosaccharide (such as glucose and fructose) that could be used as carbon source to maintain cell growth while producing NDP-sugars. Nevertheless, the kinase pathway is still the most widely used route in both *in vitro* and *in vivo* systems to generate NDP-sugars, not only because many promiscuous kinases are discovered, such as galactokinase (Zou et al., 2012) and *N*-acetylhexosamine kinase (Nishimoto and Kitaoka, 2007), but also many NDP-sugars are produced from this pathway, such as dTDP-D-glucose and UDP-*N*-acetylglucosamine, which can be used as the intermediates to further generate other NDP-sugars (Kim et al., 2012b). Therefore, in the following section, our primary focus is on the kinase pathway, which plays a crucial role in synthesizing various NDP-sugars.

Fig. 5

The ability of microorganisms such as *E. coli* to naturally synthesize diverse endogenous nucleotide sugars provides a significant advantage for the biosynthesis of various glycosides in engineered microbes. This eliminates the need to purchase expensive nucleotide sugars for glycoside production. Extracellular glucose is transported into cells via glucose facilitator diffusion protein (Glf) and subsequently phosphorylated to G-6-P by hexokinase (Glk), an important precursor for various NDP-sugars. G-6-P is converted into G-1-P by phosphoglucomutase (Pgm). Moreover, G-1-P can be produced directly from glucose by anomeric hexose kinase (Ahk). Then, G-1-

P and uridine triphosphate (UTP) are condensed to form UDP-glucose with the help of G-1-P uridylyltransferase (GalU or UgpA). Most NDP-sugars are synthesized from three biosynthetic pathways: (1) formation of UDP-sugars through the UDP-glucose pathway, which starts from G-1-P via G-1-P uridylyltransferase (GalU), including UDP-D-glucose, UDP-D-galactose, UDP-D-glucuronic acid, UDP-D-galacturonate, UDP-D-xylose, UDP-D-arabinose, UDP-L-rhamnose, and UDP-4-deoxy-4-formamido-L-arabinose; (2) synthesis of dTDP-sugars through the dTDP-glucose pathway, which are also biosynthesized from G-1-P but by the function of G-1-P thymidylyltransferase (Tgs), including dTDP-L-rhamnose, dTDP-6-deoxy-L-talose, dTDP-6-deoxy-D-allose, dTDP-4-amino 4,6-dideoxy-D-galactose, and dTDP-3-amino 3,6-dideoxy-D-galactose; (3) formation of amino UDP-sugars *via* the fructose-6-phosphate pathway. This starts from G-6-P by G-6-P isomerase (Pgi), and G-6-P can be converted into UDP-*N*-acetyl-glucosamine and its many derivatives (Fig. 6).

Fig. 6

The UDP-glucose pathway is the most extensively investigated route for the biosynthesis of various common UDP-hexoses and UDP-pentoses. This pathway begins with UDP-glucose that is further modified to produce UDP-galactose, UDP-glucuronic acid, UDP-arabinose, and UDP-xylose through various biosynthetic steps. The conjugation between UDP-sugar and the aglycon leads to the formation of various glycosides. Additionally, UDP-glucose can be epimerized to UDP-galactose by the catalysis of UDP-glucose 4-epimerase (GalE). UDP-Glucose can also be oxidized to UDP-glucuronic acid by UDP-glucose dehydrogenase (Ugd), which can be further

converted to UDP-xylose and UDP-arabinose by UDP-glucuronic acid decarboxylase (CalS9) and UDP-xylose 4-epimerase (Uxe), respectively. Moreover, UDP-glucuronic acid can also be converted to UDP-galacturonate by UDP-glucuronic acid 4-epimerase (Gla) and even amino sugars like UDP-4-deoxy-4-formamido-L-arabinose (UDP-L-Ara4FN) with the help of UDP-L-Ara4N formyltransferase/UDP-GlcA C-4"-decarboxylase (ArnA) and UDP-l-Ara4O C-4" transaminase (ArnB). By the action of UDP-rhamnose synthase (Rhm), UDP-rhamnose is generated from UDP-glucose; however, this pathway only exists in plants, not bacteria (Fig. 7).

Fig. 7

dTDP-Glucose is synthesized from G-1-P by G-1-P thymidylyltransferase (Tgs) for the synthesis of other dTDP-sugars, including dTDP-rhamnose, dTDP-talose, dTDP-allose, and dTDP-deoxyaminose, which are all biosynthesized from the intermediate dTKDG. Rhamnosides, with the rhamnose moiety from dTDP-rhamnose attached on various aglycones, are the most well-studied due to their diversity in nature. First, dTDP-glucose can be converted into dTDP-rhamnose by dTDP-glucose 4,6-dehydratase (Dh), dTDP-4-keto-6-deoxyglucose 3,5-epimerase (Epi), and dTDP-glucose 4-ketoreductase (Kr). When Kr is replaced by Tll, which encodes dTDP-6-deoxy-L-lyxo-4-hexulose reductase, dTDP-6-deoxy-L-talose is formed. dTDP-Glucose can also be transformed into dTDP-6-deoxy-allose by the action of GerFK, encoding both dTDP-hexose 3-epimerase and dTDP-4-keto-6-deoxyglucose reductase. In addition, dTDP-glucose can be used as the precursor for the biosynthesis of various dTDP-deoxyamino sugars such as dTDP-fucosamine by relative enzymes, including

dTDP-4-dehydro-6-deoxy-D-glucose-4-aminotransferase (GerB, WecE, and RffA), dTDP-6-deoxy-D-hex-4-ulose isomerase (FdtA), and dTDP-6-deoxy-D-xylohex-3-ulose aminase (FdtB) (Fig. 8).

Fig. 8

The fructose-6-phosphate pathway can synthesize the nucleotide amino sugar UDP-*N*-acetyl-glucosamine by the consecutive actions of fructose-6-phosphate transaminase (GlmS), phosphoglucosamine mutase (GlmM), glucosamine-1-phosphate *N*-acetyltransferase and *N*-acetylglucosamine-1-phosphate uridyltransferase (GlmU). UDP-*N*-Acetyl-glucosamine can be transformed into UDP-*N*-acetyl-glucosaminuronate by UDP-*N*-acetyl-glucosamine dehydrogenase (WbpA) or UDP-*N*-acetyl-galactosaminuronate by UDP-*N*-acetylglucosamine 4-epimerase (GalE2) and UDP-*N*-acetyl-galactosamine dehydrogenase (WbpO). UDP-*N*-Acetyl-glucosaminuronate can be further converted into UDP-*N*-acetyl-xylosamine by UDP-*N*-acetylxylosamine synthase (UXNAcS). Moreover, the intermediate UDP-*N*-acetyl-glucosamine can also be converted into UDP-quinovosamine by UDP-*N*-acetylglucosamine 4,6-dehydratase (Pdeg) and UDP-4-reductase (Preq). Fructose-6-phosphate can also be converted into fructose 1,6-diphosphate by fructose-6-phosphate-1-phosphotransferase (PfkA) to be further metabolized in the Embden-Meyerhof-Parnas pathway (Fig. 9).

Fig. 9

E. coli contains various nucleotide diphosphate (NDP)-sugars that play a critical role in the biosynthesis of cell wall components and other structural elements. However,

physiological concentrations of natural NDP-sugars in the cells are generally low and they are mainly for cell growth and maintenance, such as cell wall peptidoglycan synthesis (Zha and Koffas, 2017). To address this limitation, microbial cells are often modified to increase the pool of NDP-sugars, which promotes the production of polyphenolic glycosides. In engineered strains, an expanded NDP-sugar pool is necessary to balance cell growth and physiology while simultaneously producing the desired glycosides. Overexpressing NDP-sugar biosynthetic genes in the engineered strain is an effective approach to producing polyphenolic glycosides as described above. Another important approach involves preventing precursors from being converted into biomass or flowing into other irrelevant pathways, instead diverting the flow of flux towards the target NDP-sugars. some representative examples include deleting the genes for G-6-P dehydrogenase (*zwf*), which diverts G-6-P to 6-phosphogluconolactone for further processing in the pentose phosphate pathway; UDP-glucose hydrolase (*ushA*), which hydrolyzes UDP-glucose into glucose and UDP; and glucose-1-phosphatase (*agp*), which redirects glucose-1-phosphate back into glucose.

4.2 Enzyme engineering of GTs

GTs can be classified into three types based on their 3D structures: GT-A, GT-B, and GT-C (Liang et al., 2015). Both GT-A and GT-B types contain a Rossmann-like (β/α/β) folded domain that binds nucleotides. However, GT-A GTs have only one Rossmann-like fold and typically require divalent cations for activity. The NDP-sugar binding region of GT-A GTs features a conserved DxD motif that binds divalent metal ions (Mg^{2+} or Mn^{2+}), which are essential for activating catalysis and stabilizing the

negative charge on the leaving group (phosphate group). On the other hand, GT-B GTs have two Rossmann-like folds and typically lack relevant conserved domains like the DxD motif. They are metal-ion-independent proteins that do not require divalent cations for activity. However, divalent cations can facilitate the product release for few GT-B type GTs (Chen et al., 2012; Larivière et al., 2003). GT-C type GTs have complex folds with multiple transmembrane α-helices and use lipid-linked sugar donors. GT-C type GTs (such as oligosaccharyltransferases) are less studied compared to the other two types (Liang et al., 2015; Moremen and Haltiwanger, 2019).

Plant UGTs belong to the GT1 family based on their amino acid sequences and are further classified as GT-B GTs (GUO et al., 2021; Yang et al., 2023). The N- and C-terminal domains of GT-B GTs bind sugar acceptor and sugar donor, respectively. These binding sites are located in the cleft region between the two domains, each of which features a central β-sheet flanked by α-helices on both sides (Wang, 2009). The C-terminal domain of GT-B GTs has a highly conserved plant secondary product glycosyltransferase (PSPG) motif which is involved in the recognition of the UDP-sugar (Chen et al., 2021). The N-terminal domain of GT-B GTs is located in a hydrophobic pocket and is imperative for structural diversity because of the loose binding with the aglycone (Yao et al., 2022). Contrary to retaining GTs, plant UGTs are inverting GTs (McGraphery and Schwab, 2020) which invert the configuration of the anomeric (C1) linkage of the NDP-sugar donor during the sugar transfer process (for example, from NDP-α-sugar to β-glycoside) by catalyzing an S_N2-like single displacement reaction (Liang et al., 2015).

The mechanisms of *O*-GTs differ from those of *C*-, *N*-, and *S*- GTs in terms of nucleophile behavior in the catalytic site (Yao et al., 2022). To form new *O*-glycosidic bonds, GTs initially bind the sugar-acceptor substrate and orient a specific hydroxyl group from the polyphenolic compound. A catalytic base deprotonates the nucleophile hydroxyl group of the sugar-acceptor substrate, typically using a histidine residue located in the N-terminal domain of most UGT structures. To stabilize the histidine and balance its charge after deprotonation of the sugar-acceptor, the adjacent aspartate residue forms a hydrogen bond with the histidine residue. Next, the nucleophile, which is the sugar-acceptor substrate with a deprotonated hydroxyl group, attacks the anomeric (C1) carbon of the sugar-donor substrate and displaces the nucleotide moiety of the leaving group from the opposite face. This process leads to an inversion of the anomeric configuration of the product (Breton et al., 2012) (Fig. 10a).

In heterologous biosynthetic pathways, wild type GTs can sometimes exhibit low catalytic activity, produce a mixture of glycosides, and have strict substrate specificity (Li et al., 2020). Additionally, many GTs are prone to poor stability and functional loss due to their high free energy state. Surface residue mutations have been shown to reduce enzyme lability and extend the enzyme's catalytic lifetime, thereby improving catalytic efficiency without altering the ability to bind substrates (Choi et al., 2021). Therefore, considering the structures and mechanism of plant UGTs, different approaches of protein engineering have been developed to facilitate UGT-mediated glycosylation (Fig. 10b), including improved catalytic activity, product regioselectivity, and expanded substrate specificity.

Fig. 10

4.2.1. Improved catalytic activity

It is commonly accepted that factors such as the size of the binding pocket are crucial for enhancing catalytic efficiency in flavonoid glycosylation. To this end, Singh et al. performed enzyme engineering on WsUGT73A16 from *Withania somnifera*. The A337C mutant exhibited around 2.6-fold improved catalytic efficiency toward the sugar-acceptor (baicalein) by increasing the stability of the PSPG motif through the formation of a disulfide bridge with C355. Similarly, the Q339A mutant showed approximately 6.8-fold increased catalytic efficiency toward sugar-donor (UDP-glucuronic acid) by enlarging the binding pocket of WsUGT73A16 (Singh et al., 2018). In another work, the quadruple VFAH mutant of MiCGT from *Mangifera indica* displayed 120-fold enhanced catalytic activity to quercetin than the wild type. Mutations at W93, F191 and R282 expanded the binding pocket, while a mutation at R282 also facilitated the deprotonation of the required substrate. The whole complex structure was stabilized by mutating V124 (Wen et al., 2021). These examples serve as valuable case studies for improving the glycosylation efficiency of native substrates, with broad implications for enhancing industrial processes that require GTs as enzymatic catalysts. Improved binding interactions between enzymes and substrates can also lead to enhanced catalytic activity. A notable example of this principle is the Q19A mutation in PaUGT1 from *Plagiochasma appendiculatum*, which exhibited a 3.4-fold increase in catalytic efficiency towards quercetin and a 0.8-fold increase towards apigenin compared to the wild type enzyme. As a result, this mutant was able

to produce flavonol 7-*O*-glucosides in *E. coli* with an over 70% substrate conversion rate. The docking analysis revealed that the deeper position of the binding pocket facilitated the bonding of the two ligands (sugar-donor and acceptor), leading to a more favorable orientation for enhanced activities (Zhu et al., 2020). The point mutation V200E of UGT85H2 from *Medicago* truncatula resulted in a significant improvement in catalytic efficiency for kaempferol (15-fold) and biochanin A (54-fold). Further studies through molecular modeling and docking demonstrated that this improvement was due to stronger interactions, specifically the formation of a hydrogen bond between residue E200 in the mutant V200E and 4'-OH of kaempferol and 7-OH of biochanin A (Modolo et al., 2009). Therefore, expanding the binding pocket and strengthening interactions between the enzyme and substrate are two main mechanisms for improving catalytic activity. Additionally, the orientation of substrates also plays a vital role in facilitating glycosides production. For example, in a crude enzyme experiment of PhUGT from *Petunia hybrida*, the F368T variant showed an enhanced conversion rate towards both quercetin (6.5-fold) and UDP-*N*-acetyl-D-galactosamine (6.5-fold). Further molecular docking studies revealed that the mutation of F368 may have influenced the orientation of UDP-N-acetyl-D-galactosamine and quercetin residue, thereby regulating the generation of the glycosylated product (Xu et al., 2022).

4.2.2. Product regioselectivity

By manipulating the entrance size and hydrophobicity of the catalytic regions, as well as strengthening interactions between the substrate and enzyme, it is possible to achieve regioselectivity in glycosylating target substrates. Li et al. successfully

generated three mutants of UGT74AC2 from *Siraitia grosvenorii* by using Focused Rational Iterative Site-specific Mutagenesis (FRISM). The three mutants, namely 3,7G-M3, 3G-M2, and 3,7G-M1, were able to control the regioselectivity of silybin A O-glycosylation. In comparison to the wild type that produced an almost equal distribution of three glycosides, these mutants produced specific glycosides with high selectivity: 3-O-glycoside (3G, 94%), 7-O-glycoside (7G, >99%), and 3,7-O-diglycoside (3,7G, >99%). By utilizing protein-ligand docking based on the crystal structure of UGT74AC2, the computational analysis has revealed that the reaction regioselectivity can be influenced by several factors, including the size of the binding pocket, steric hindrance, and hydrophobicity. In the 3G-WT model, the steric hindrance between residue Y392 and silybin was observed. To address this issue and expand the binding pocket, the Y392 residue was substituted with a smaller threonine residue. Regioselective production of silybin 3-*O*-glycoside was improved through hydrophobic interaction with the A ring of silybin by introducing a T149V mutation in the 3G-M3 model. Regioselective production of silybin 7-*O*-glycoside was achieved in the 3G-M2 model. By enlarging residue 200 in UGT74AC2, we were able to decrease the size of the active pocket, thereby preventing silybin from reorienting within the binding site. the proximity of the phenol group in residue Y11 to the D ring of silybin 7-*O*-glycoside compensated for π-π interactions, thus promoting the production of silybin 3,7-*O*-diglycoside (Li, J. et al., 2021). Besides the size of the binding pocket, steric hindrance can also affect the regioselectivity of glycoside production. Li et al. achieved the production of flavonoid disaccharides by introducing mutations to GT1 from

Cyclocarya paliurus. They removed the extra *β*-sheet that functions as a cap to prevent the substrate from entering the binding pocket by deleting the V309-320 residues (Li et al., 2019). These findings demonstrate that enzyme engineering of UGTs is an effective means of producing specific polyphenolic glycosides, rather than a mixture of related products. Additionally, reducing steric hindrance at the entrance of the catalytic domain is a promising technique for producing relevant glycosides.

4.2.3. Expanded substrate specificity

Broadening both acceptor and donor substrate specificity is an effective enzymatic tool for producing a wide range of polyphenolic glycosides. Recent examples of successful enzyme engineering have provided a blueprint for the development of this technique. For instance, a benchmark study involved the point mutation of Cys142 in PaGT2 from *Phytolacca americana*, which expanded its substrate specificity from its native substrate piceatannol to include resveratrol. Specifically, two structure-guided point mutations, namely, C142A and C142F, guided the regioselectivity for the production of resveratrol 4′-*O*-β-glucoside or resveratrol 3-*O*-β-glucoside, respectively (Maharjan et al., 2020). This study demonstrates that crystal structure and molecular docking of GTs could facilitate mutagenesis studies.

Interestingly, shrinking the binding pocket may switch target sugar acceptor types of GTs. For instance, three mutants G87F, I199F, and L204F of SrUGT76G1 from *Stevia rebaudiana* change the sugar acceptor substrate from diterpene to flavonoid (Liu et al., 2020). Broadening the substrate specificity of donor molecules can be an effective strategy for reducing costs, as some sugar donor species may be rare or expensive.

In addition to broadening acceptor substrate specificity, various enzyme engineering approaches have been used to widen the substrate specificity of donor molecules. Structural and mutational analysis of UGT89C1 from *A. thaliana* indicated that His357 is one of four main residues involved in the recognition of sugar donor UDP-β-L-rhamnose. The site-directed mutagenesis of the histidine residue (H357Q) in the donor binding site of UGT89C1 enabled the enzyme to utilize UDP-glucose instead of UDP-β-L-rhamnose as the sugar donor (Zong et al., 2019). Amino acid exchanges between two GTs may also expand the range of sugar donors. For example, AtUGT78D2 and AtUGT78D3 from *A. thaliana* use UDP-glucose and UDP-arabinose, respectively. Through amino acid exchanges between AtUGT78D2 (76, 228, and 336) and AtUGT78D3 (73, 225, and 335), AtUGT78D2 acquired the ability to utilize UDP-arabinose. Further molecular modeling studies have shown that mutating methionine to alanine at position 288 is responsible for the observed effect (Kim et al., 2013). Therefore, endogenous donor sugars in the host cell are important materials for whole cell biocatalysts, and mutants of GTs with expanded sugar donor substrate specificity can facilitate the production of bioactive glycosides.

4.2.4 Other properties

In some cases, GT engineering strategies have yielded enzymes with entirely modified glycosylation properties, providing a distinct range of functionalities. He et al. elucidated the crystal structure of TcCGT1 from *Trollius chinensis* that has broad substrate specificity. Site-directed mutagenesis conducted at two residues (I94E and G284K) in the acceptor site changed the glycosylation pattern from *C*-glycosylation to

O-glycosylation of flavonoids (He et al., 2019). It is interesting that point mutations may swithch enzyme's activity from glycosyltransferase to glucosidase. The single mutation V200E of UGT85H2 from *Medicago truncatula* converted kaempferol 3-*O*-glucoside into kaempferol. The molecular docking study showed that the acidic residue Glu200 initiates the reverse reaction, and its side chain plays a role in extending it to the deglycosylation region of the enzyme (Modolo et al., 2009). Further theoretical work is required to fully comprehend the correlation between specific residues and their functions on GTs, as well as to investigate how engineering strategies can be used to manipulate glycobiology processes. The examples discussed above demonstrate the wide range of glycosylation property changes that can be achieved through enzyme engineering strategies.

While structural studies are crucial in explaining the catalytic and property changes of engineered GTs, obtaining the crystal structure of GTs to understand the key residues remains a time-consuming and challenging process. The emerging tools such as AlphaFold could provide some help in predicting enzyme structures for rational engineering of GTs. Homology modeling and molecular docking between GTs and substrates, along with structure-guided directed evolution methods such as FRISM and iterative saturation mutagenesis (ISM), offer promising approaches to achieving precise mutations at critical amino acid residues for desirable enzyme properties (Akere et al., 2020). While pertinent engineering studies have shown similar trends in enhancing GT properties, it is worth noting that mutating specific residues outside of the active sites, such as the binding pocket or PSPG motif, can also improve GT activities (Li, J. et al.,

2020). Moreover, it was found that catalytic bases during glycosylation are not always histidine or aspartate residues (Noguchi et al., 2007). As a result, new techniques are required to track the dynamic changes of GT structure during glycosylation, allowing a more thorough exploration of the unique mechanisms of GTs. This, in turn, will facilitate protein engineering for the creation of new GTs with more customized functions. With the help of diverse and highly efficient GTs, it is expected that large-scale industrial applications of bioactive polyphenolic glycosides can be achieved in the foreseeable future.

5. Conclusions and Perspectives

Phytochemicals, including polyphenolic compounds, are important secondary metabolites, and their biological activities can be modulated by sugar moieties (De Bruyn et al., 2015b). When engineering the biosynthesis of plant-derived glycosides in microbes, it is noteworthy that the biosynthetic mechanisms and types of NDP-sugars can vary between plants and bacteria. For example, plants and bacteria can form different nucleotide-rhamnoses. Specifically, UDP-glucose can be directly transformed into UDP-rhamnose by rhamnose synthase (RHM) in plants. By contrast, three enzymes are needed to convert dTDP-glucose into dTDP-rhamnose in bacteria (Reiter, 2008). What's more, both plants and bacteria possess unique NDP-sugar biosynthetic pathways. For instance, bacteria have a distinctive nucleotide sugar biosynthetic pathway that begins with glucosamine-1-phosphate, which is a precursor to UDP-*N*-acetylglucosamine, whereas plants do not have this pathway (Samuel and Reeves, 2003). Enzymes required for biosynthesizing bacterial nucleotide sugar derivatives are also exclusive to bacteria (De Bruyn et al., 2015b). Nevertheless, UDP-apiose synthase, which converts UDP-glucuronate into UDP-apiose, is only present in plants (De Bruyn

et al., 2015b). Therefore, to further expand the repertoire of health-benefiting and novel polyphenolic glycosides, it is critical to combine biosynthetic tools from various sources including plants, microbes, and even animals. For example, we are presently investigating the generation of mono- and di-glycosides of the anti-tuberculosis agent chlorflavonin for improved bioavailability by sequentially overexpressing two different GTs from both plant and microbial sources (Rehberg et al., 2018). To achieve this goal, we will utilize two recently characterized microbial GTs that exhibit broad substrate specificity in combination with versatile plant glycosyltransferases from *A. thaliana* (Ren et al., 2022a; Ren et al., 2022c).

Biological glycosylation produces less complex mixtures compared to chemical synthesis and allows for improved control of the regioselectivity and stereoselectivity of target glycosides (Gantt et al., 2011). However, the diversity of NDP-sugar biosynthetic pathways in microbes is a double-edged sword, as certain GTs may exhibit promiscuity and produce unwanted byproducts. For example, two byproducts, quercetin-3-O-glucose and quercetin-3-O-N-acetylglucosamine were produced when Cho et al. attempted the biosynthesis of quercetin-3-O-N-acetylxylosamine (Cho et al., 2016b). The concentration of endogenous NDP-sugars plays a crucial role in the final production titer of target glycosides. To minimize undesired byproducts, two possible approaches can be pursued. Firstly, researchers can manipulate the nucleotide sugar biosynthetic pathways to enhance the supply of the desired NDP-sugar by deleting the genes in the competing pathways. Alternatively, directed evolution of GTs can be performed to enhance their catalytic efficiency on different substrates and improve their specificity on both sugar donors and acceptors (Osmani et al., 2008). Reducing the metabolic burden in engineered strains due to de novo biosynthesis of target polyphenolic glycosides is another long-term issue to address. An effective approach is

to insert heterologous genes into the host genome, as demonstrated in the production of cyanogenic glycoside dhurrin in yeast (Kotopka and Smolke, 2019). CRISPR/Cas9 genome editing can also be used for gene deletion or insertion to facilitate the production of polyphenolic glycosides in microbes (Moon et al., 2020).

Many GTs, including those from A. thaliana, have been characterized for biosynthesizing glycosides. However, future work should aim to explore novel GTs from new sources, especially microbes, to expand the enzyme toolbox for glycosylation. Microbial GTs often possess broad substrate specificity, offering a more convenient method for producing diverse glycosides for drug candidate and pro-drug development. Further understanding of microorganisms' biosynthetic machinery will allow rational engineering for the efficient production of corresponding glycosides. For example, while *E. coli* is commonly used for producing polyphenolic glycosides, *S. cerevisiae* has also been successfully engineered as a platform strain for glycoside production by deleting endogenous glucosidases and rewiring the metabolic flux to desired products (Wang et al., 2016). Research has shown that different quercetin glycosides are produced when expressing the same GT in *E. coli* and *S. cerevisiae* (Ren et al., 2022c). Thus, exploring different microbial hosts to express GTs is also useful for generating diverse glycosides.

Various methods have been developed to produce polyphenolic glycosides in microbes and even plants, including manipulation of endogenous metabolic pathways, overexpression of heterologous genes, and site-directed mutagenesis of dedicated GTs. However, most of the studies discussed in this review were performed at the laboratory scale, often in flasks or benchtop bioreactors. Although some polyphenolic glycosides have been produced at gram scale, most of the current examples are still at milligram

or sub-milligram scale, which is not practical for industrial processes. Therefore, future industrial production of these compounds will depend heavily on continued research to improve growth and production efficiency, including the development of efficient substrate influx and product efflux in engineered strains, identification of transporters for intermediates in polyculture systems, enzyme evolution, and fermentation optimization. While some common phenolic glycosides such as glucosides and rhamnosides have been studied for their bioactivities, there is still much to be explored regarding the health benefits of less common glycosides such as allosides, talosides, deoxyaminosides, and glucosaminosides, which will help understand how glycosylation affects their biological activities. Additionally, the glycosylation of lignans and uncommon flavonoids such as chalcones and neoflavones is an interesting area for future research. It is important to note that the antioxidant activity of polyphenolic glycosides has mainly been determined through *in vitro* studies (Williams et al., 2004), and further *in vivo* studies are necessary to fully understand their potential health benefits.

In summary, polyphenolic glycosides represent a large group of bioactive molecules with a diversity of health benefits and medicinal properties. Engineered production of polyphenolic glycosides in microbes represents a promising way to manufacture these valuable compounds in a cost-effective and sustainable way.

Author contribution

All authors were involved in the preparation of the manuscript.

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Declaration of Competing Interest

Authors declare that there is no conflict of interest.

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Figure Captions

Fig. 1. Classification of plant polyphenolic compounds and representative structures. The compounds in the parentheses are the typical examples of each subgroup. The compounds in green boxes are included and described in the engineered production section of this review.

Fig. 2. The effects of glycosylation on polyphenolic compounds. (a) Carbohydrate-active enzymes for *in vitro* glycosylation; (b) General engineering strategy to generate polyphenolic glycosides *in vivo*; (c) Biological properties of polyphenolic compounds mediated by glycosylation; (d) Absorption and metabolism of dietary polyphenolic glycosides.

Fig. 3. Methods of obtaining polyphenolic glycosides.

Fig. 4. Biosynthetic approaches to producing bioactive polyphenolic glycosides. (a) Five main strategies to engineer the metabolic pathway in microorganisms for biosynthesizing bioactive polyphenolic glycosides. (b) Key advantages and disadvantages of five methods for producing polyphenolic glycosides in microbes.

Fig. 5. Three main pathways for common NDP-sugars. NTP: Nucleotide triphosphate; NDP: Nucleotide diphosphate.

Fig. 6. Nucleotide sugar pathways for the biosynthesis of different glycosylated polyphenols. The sugars present in the green rectangular boxes are the starter substrates. Three blue ovals indicate three important branches. Three pathways present in the yellow rectangular boxes are common routes for generating NDP-sugars with typical examples. The sugar present in the pink box is a common intermediate for various NDP-

sugars. Representative gene deletions are marked with “x.” Glf: Glucose facilitator diffusion protein; Ahk: Anomeric hexose kinase; Glk: Hexokinase; Zwf: G-6-P dehydrogenase; Pgi: G-6-P isomerase; Pgm or Nfa44530: Phosphoglucomutase; Agp: Glucose 1-phosphatase; UshA: UDP-glucose hydrolase; Tgs: G-1-P thymidylyltransferase (nucleotidylyltransferase); GalU or UgpA: G-1-P uridylyltransferase (nucleotidylyltransferase); SUS: Sucrose synthase; BaSP: Sucrose phosphorylase; CBP: Cellobiose phosphorylase; PyrE: Orotate phosphoribosyltransferase; PyrF: Orotidine-5'-phosphate decarboxylase; PyrH: Uridylate kinase; NDK: Nucleoside diphosphate kinase.

Fig. 7. Biosynthetic pathways of various UDP-sugars from UDP-glucose. The sugar in the blue oval is the starting precursor. The sugar present in the pink box is the important intermediate for various UDP-sugars. Green arrows indicate the last step to biosynthesize glycosides by various GTs from different sources. The dashed arrow indicates the pathway only existing in plants and is not present in the bacteria. Rhm or MUM4: UDP-Rhamnose synthase; GalE or UGE: UDP-glucose 4-epimerase; Ugd or CalS8: UDP-glucose dehydrogenase; Gla: UDP-glucuronic acid 4-epimerase; CalS9: UDP-glucuronic acid decarboxylase; UXS: UDP-xylose synthase; Uxe: UDP-xylose 4-epimerase; ArnA: UDP-L-Ara4N formyltransferase/UDP-GlcA C-4"-decarboxylase; ArnB: UDP-L-Ara4O C-4" transaminase.

Fig. 8. Biosynthetic pathways of various dTDP-sugars from dTDP-glucose. The sugar in the blue oval is the starting precursor. The sugar present in the pink box is a common intermediate for various TDP-sugars. Green arrows are the last step to

biosynthesize glycosides by various GTs. Dh: dTDP-glucose 4,6-dehydratase; Epi: dTDP-4-keto-6-deoxyglucose 3,5-epimerase; Kr: dTDP-glucose 4-ketoreductase; RfbD: dTDP-4-dehydrorhamnose reductase; Tll: dTDP-6-deoxy-L-lyxo-4-hexulose reductase; GerB, WecE, and RffA: dTDP-4-dehydro-6-deoxy-D-glucose-4-aminotransferase; FdtA: dTDP-6-deoxy-D-hex-4-ulose isomerase; FdtB: dTDP-6-deoxy-D-xylohex-3-ulose aminase; GerFK: dTDP-hexose 3-epimerase and dTDP-4-keto-6-deoxyglucose reductase.

Fig. 9. Biosynthetic pathways of UDP-sugars from D-fructose-6-phosphate. The sugar in the blue oval is the starting precursor. The sugar present in the pink box is the intermediate for various UDP-sugars. Green arrows are the last step to biosynthesize glycosides by various GTs. PfkA: Fructose-6-phosphate 1-phosphotransferase; GlmS: Fructose-6-phosphate transaminase; GlmM: Phosphoglucosamine mutase; GlmU: Glucosamine-1-phosphate *N*-acetyltransferase and *N*-acetylglucosamine-1-phosphate uridyltransferase; GalE2: UDP-*N*-acetylglucosamine 4-epimerase; WbpO: UDP-*N*-acetyl-D-galactosamine dehydrogenase; WbpA: UDP-*N*-acetyl-D-glucosamine dehydrogenase; UXNAcS: UDP-*N*-acetylxylosamine synthase; Pdeg: UDP-*N*-acetylglucosamine 4,6-dehydratase; Preq: UDP-4-reductase.

Fig. 10. Enzyme engineering of GTs for production of polyphenolic glycosides. (a) Catalytic mechanism of *O*-GTs. (b) Common strategies for GT engineering.

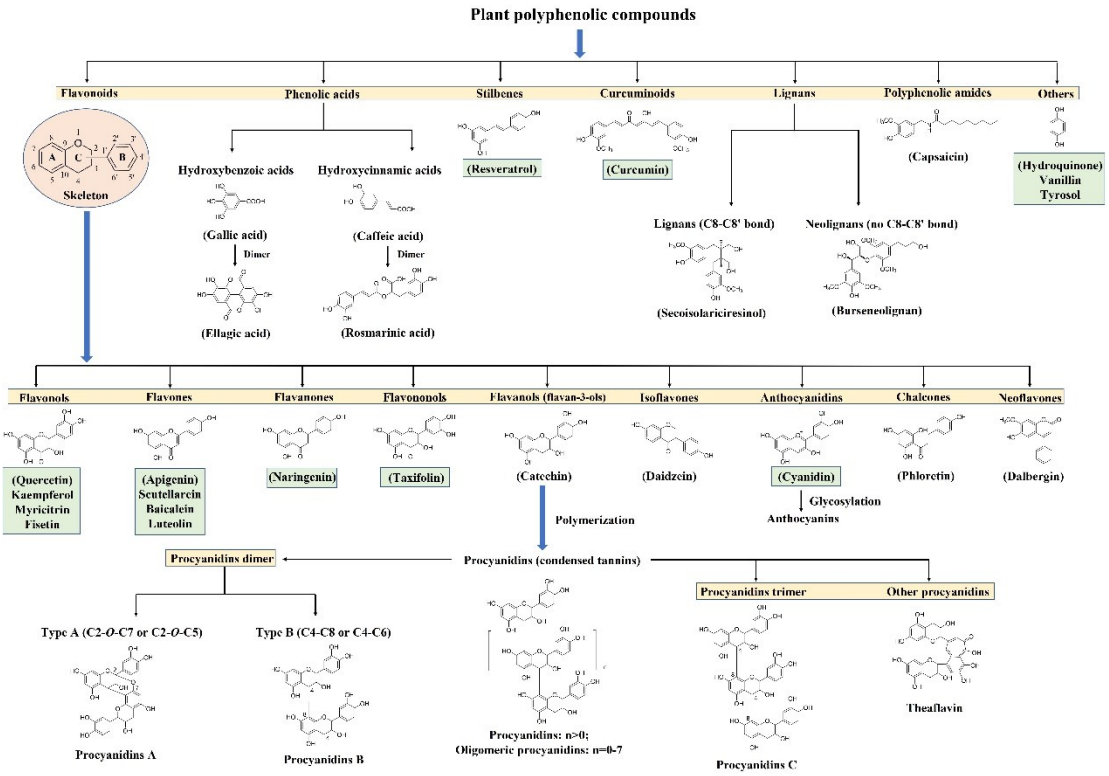

Plant polyphenolic compounds
Flavonoids
Phenolic acids
Stilbenes
Curcuminoids
Lignans
Polyphenolic amides
Others
Skeleton
Hydroxybenzoic acids
Hydroxycinnamic acids
(Resveratrol)
(Curcumin)
(Capsaicin)
(Hydroquinone)
Vanillin
Tyrosol
(Gallic acid)
(Caffeic acid)
Dimer
Dimer
Lignans (C8-C8' bond)
Neolignans (no C8-C8' bond)
(Ellagic acid)
(Rosmarinic acid)
(Secoisolariciresinol)
(Burseneolignan)
Flavonols
Flavones
Flavanones
Flavononols
Flavanols (flavan-3-ols)
Isoflavones
Anthocyanidins
Chalcones
Neoflavones
(Quercetin)
Kaempferol
Myricitrin
Fisetin
(Apigenin)
Scutellarein
Baicalein
Luteolin
(Naringenin)
(Taxifolin)
(Catechin)
(Daidzein)
(Cyanidin)
(Phloretin)
(Dalbergin)
Glycosylation
Anthocyanins
Polymerization
Procyanidins dimer
Procyanidins (condensed tannins)
Procyanidins trimer
Other procyanidins
Type A (C2-O-C7 or C2-O-C5)
Type B (C4-C8 or C4-C6)
Procyanidins A
Procyanidins B
Procyanidins: n>0;
Oligomeric procyanidins: n=0-7
Theaflavin
Procyanidins C

Figure 1

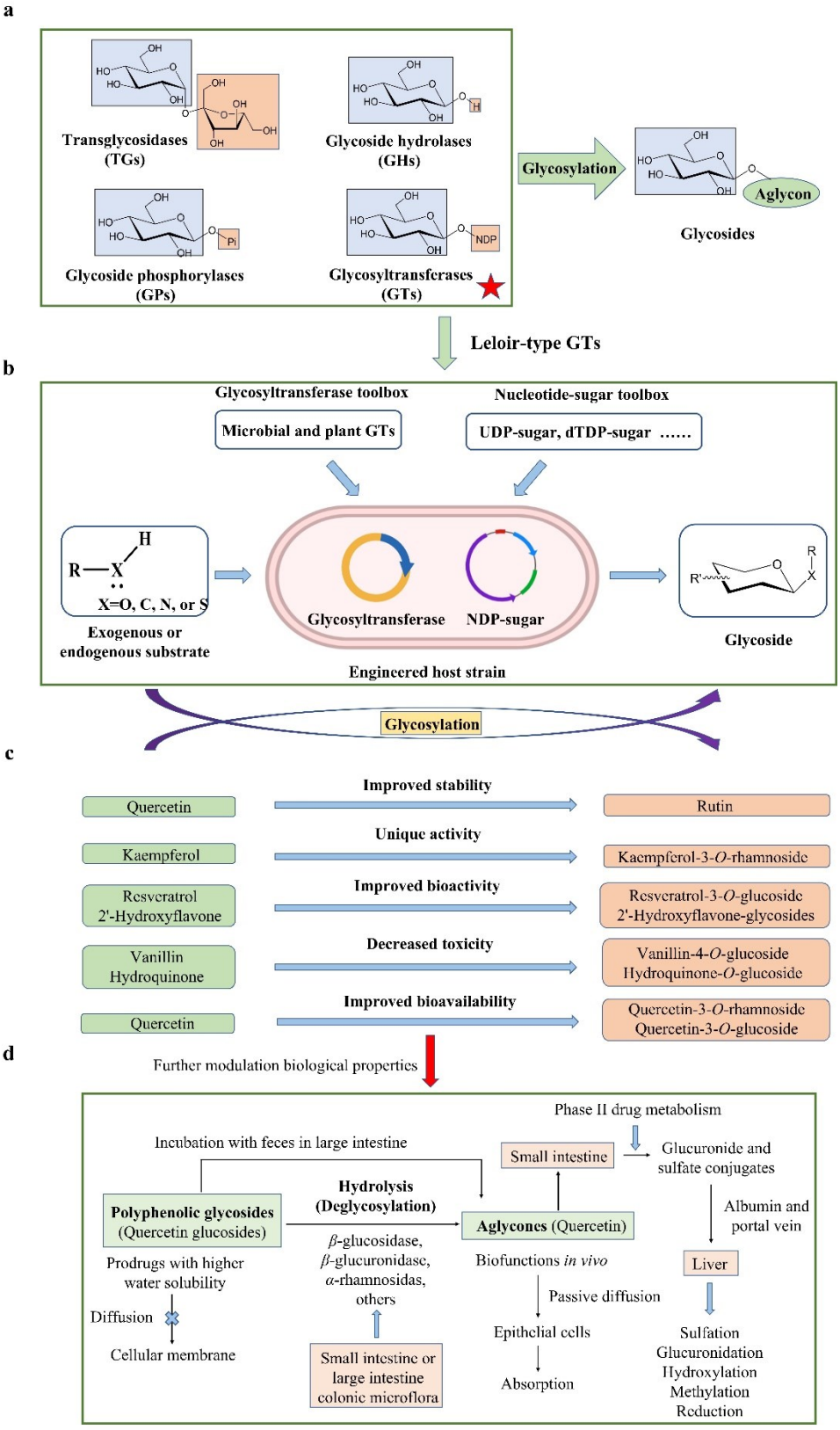

a
Transglycosidases (TGs)
Glycoside hydrolases (GHs)
Glycosylation
Aglycon
Glycosides
Glycoside phosphorylases (GPs)
Glycosyltransferases (GTs)
Leloir-type GTs
b
Glycosyltransferase toolbox
Nucleotide-sugar toolbox
Microbial and plant GTs
UDP-sugar, dTDP-sugar ……
X=O, C, N, or S
Exogenous or endogenous substrate
Glycosyltransferase
NDP-sugar
Glycoside
Engineered host strain
Glycosylation
c
Improved stability
Quercetin
Rutin
Unique activity
Kaempferol
Kaempferol-3-O-rhamnoside
Improved bioactivity
Resveratrol
2'-Hydroxyflavone
Resveratrol-3-O-glucoside
2'-Hydroxyflavone-glycosides
Decreased toxicity
Vanillin
Hydroquinone
Vanillin-4-O-glucoside
Hydroquinone-O-glucoside
Improved bioavailability
Quercetin
Quercetin-3-O-rhamnoside
Quercetin-3-O-glucoside
d
Further modulation biological properties
Phase II drug metabolism
Incubation with feces in large intestine
Small intestine
Glucuronide and sulfate conjugates
Albumin and portal vein
Polyphenolic glycosides (Quercetin glucosides)
Hydrolysis (Deglycosylation)
Aglycones (Quercetin)
β-glucosidase, β-glucuronidase, α-rhamnosidas, others
Prodrugs with higher water solubility
Biofunctions in vivo
Liver
Passive diffusion
Diffusion
Epithelial cells
Sulfation
Glucuronidation
Hydroxylation
Methylation
Reduction
Cellular membrane
Small intestine or large intestine colonic microflora
Absorption

Figure 2

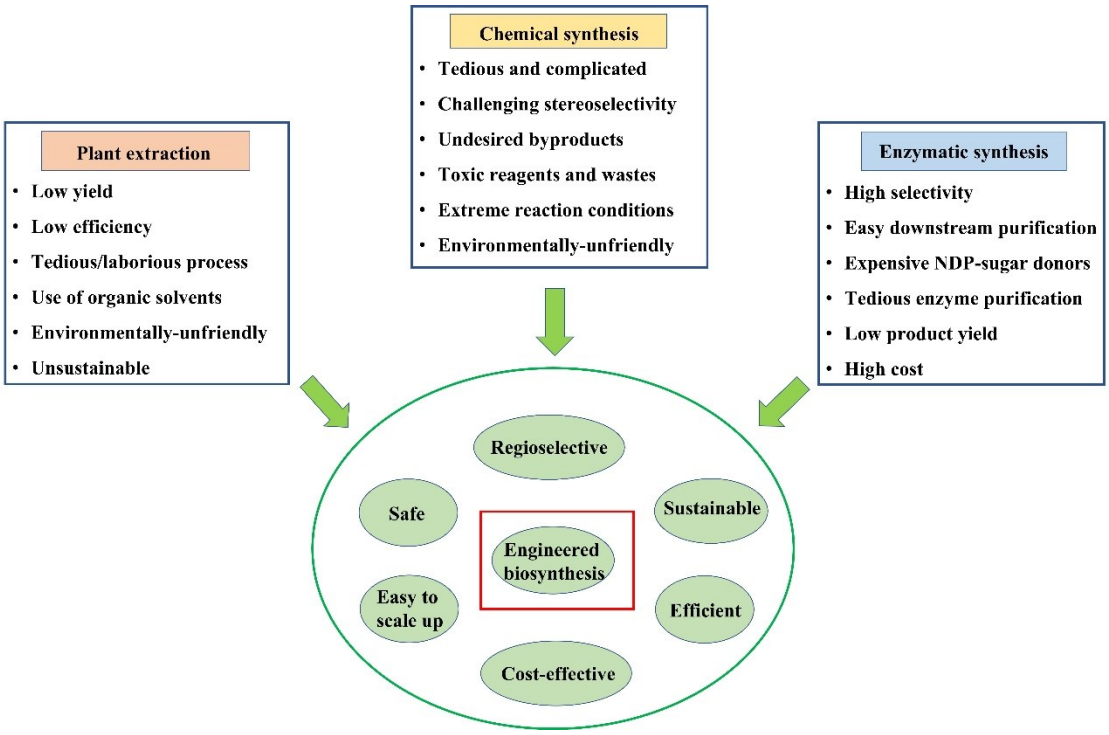

Chemical synthesis
• Tedious and complicated
• Challenging stereoselectivity
• Undesired byproducts
• Toxic reagents and wastes
• Extreme reaction conditions
• Environmentally-unfriendly
Plant extraction
• Low yield
• Low efficiency
• Tedious/laborious process
• Use of organic solvents
• Environmentally-unfriendly
• Unsustainable
Enzymatic synthesis
• High selectivity
• Easy downstream purification
• Expensive NDP-sugar donors
• Tedious enzyme purification
• Low product yield
• High cost
Regioselective
Safe
Sustainable
Engineered biosynthesis
Easy to scale up
Efficient
Cost-effective

Figure 3

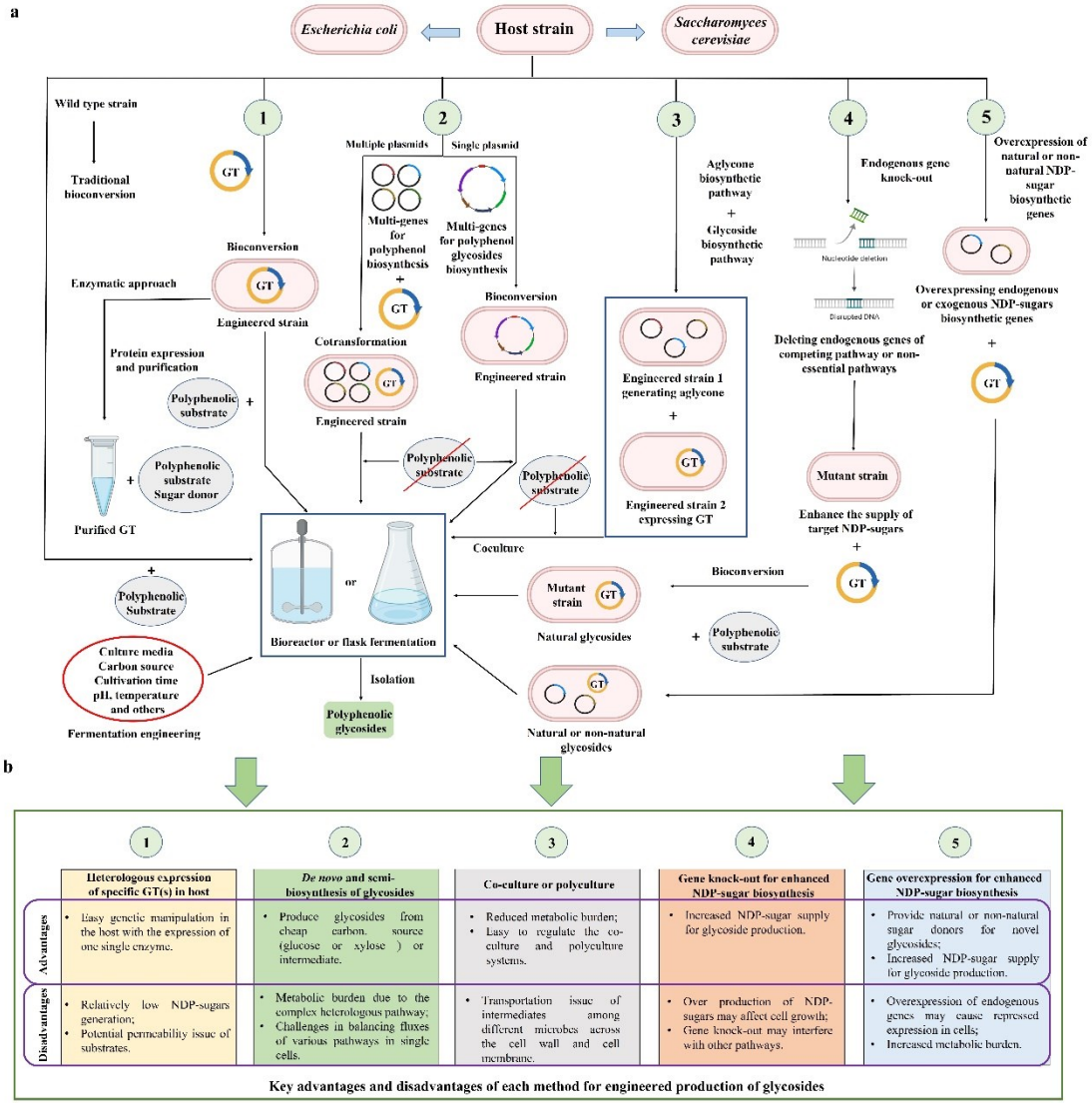

a
Escherichia coli
Host strain
Saccharomyces cerevisiae
Wild type strain
Traditional bioconversion
1
2
3
4
5
Multiple plasmids
Single plasmid
Aglycone biosynthetic pathway
+
Glycoside biosynthetic pathway
Endogenous gene knock-out
Overexpression of natural or non-natural NDP-sugar biosynthetic genes
Bioconversion
Enzymatic approach
Engineered strain
Multi-genes for polyphenol biosynthesis
+
Multi-genes for polyphenol glycosides biosynthesis
Cotransformation
Bioconversion
Engineered strain
Engineered strain 1 generating aglycone
+
Engineered strain 2 expressing GT
Nucleotide deletion
Disrupted DNA
Deleting endogenous genes of competing pathway or non-essential pathways
Overexpressing endogenous or exogenous NDP-sugars biosynthetic genes
+
Protein expression and purification
Polyphenolic substrate
+
Polyphenolic substrate
Sugar donor
Purified GT
Polyphenolic substrate
Polyphenolic substrate
Coculture
Mutant strain
Enhance the supply of target NDP-sugars
+
Polyphenolic Substrate
Culture media
Carbon source
Cultivation time
pH, temperature
and others
Fermentation engineering
or
Bioreactor or flask fermentation
Isolation
Polyphenolic glycosides
Mutant strain
Natural glycosides
Bioconversion
+
Polyphenolic substrate
Natural or non-natural glycosides
b
1
2
3
4
5
Heterologous expression of specific GT(s) in host
De novo and semi-biosynthesis of glycosides
Co-culture or polyculture
Gene knock-out for enhanced NDP-sugar biosynthesis
Gene overexpression for enhanced NDP-sugar biosynthesis
Advantages
Easy genetic manipulation in the host with the expression of one single enzyme.
Produce glycosides from cheap carbon source (glucose or xylose) or intermediate.
Reduced metabolic burden;
Easy to regulate the co-culture and polyculture systems.
Increased NDP-sugar supply for glycoside production.
Provide natural or non-natural sugar donors for novel glycosides;
Increased NDP-sugar supply for glycoside production.
Disadvantages
Relatively low NDP-sugars generation;
Potential permeability issue of substrates.
Metabolic burden due to the complex heterologous pathway;
Challenges in balancing fluxes of various pathways in single cells.
Transportation issue of intermediates among different microbes across the cell wall and cell membrane.
Over production of NDP-sugars may affect cell growth;
Gene knock-out may interfere with other pathways.
Overexpression of endogenous genes may cause repressed expression in cells;
Increased metabolic burden.
Key advantages and disadvantages of each method for engineered production of glycosides

Figure 4

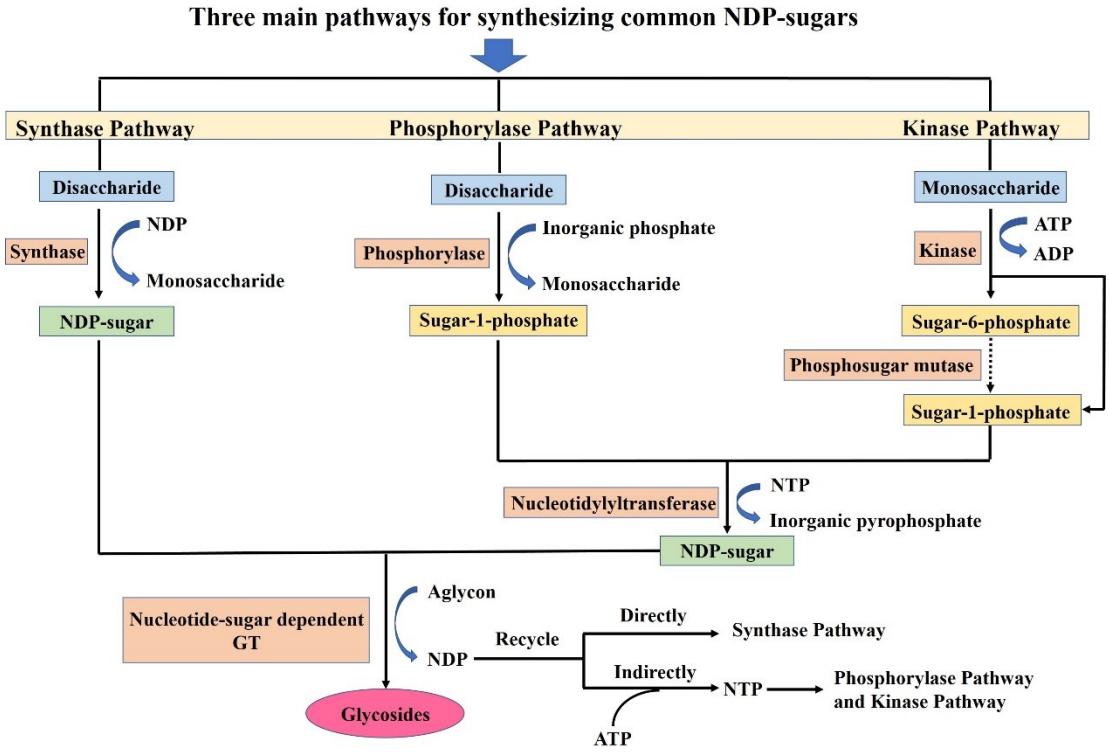

Three main pathways for synthesizing common NDP-sugars
Synthase Pathway
Phosphorylase Pathway
Kinase Pathway
Disaccharide
Disaccharide
Monosaccharide
Synthase
NDP
Monosaccharide
Phosphorylase
Inorganic phosphate
Monosaccharide
Kinase
ATP
ADP
NDP-sugar
Sugar-1-phosphate
Sugar-6-phosphate
Phosphosugar mutase
Sugar-1-phosphate
Nucleotidylyltransferase
NTP
Inorganic pyrophosphate
NDP-sugar
Nucleotide-sugar dependent GT
Aglycon
NDP
Recycle
Directly
Synthase Pathway
Indirectly
NTP
Phosphorylase Pathway and Kinase Pathway
ATP
Glycosides

Figure 5

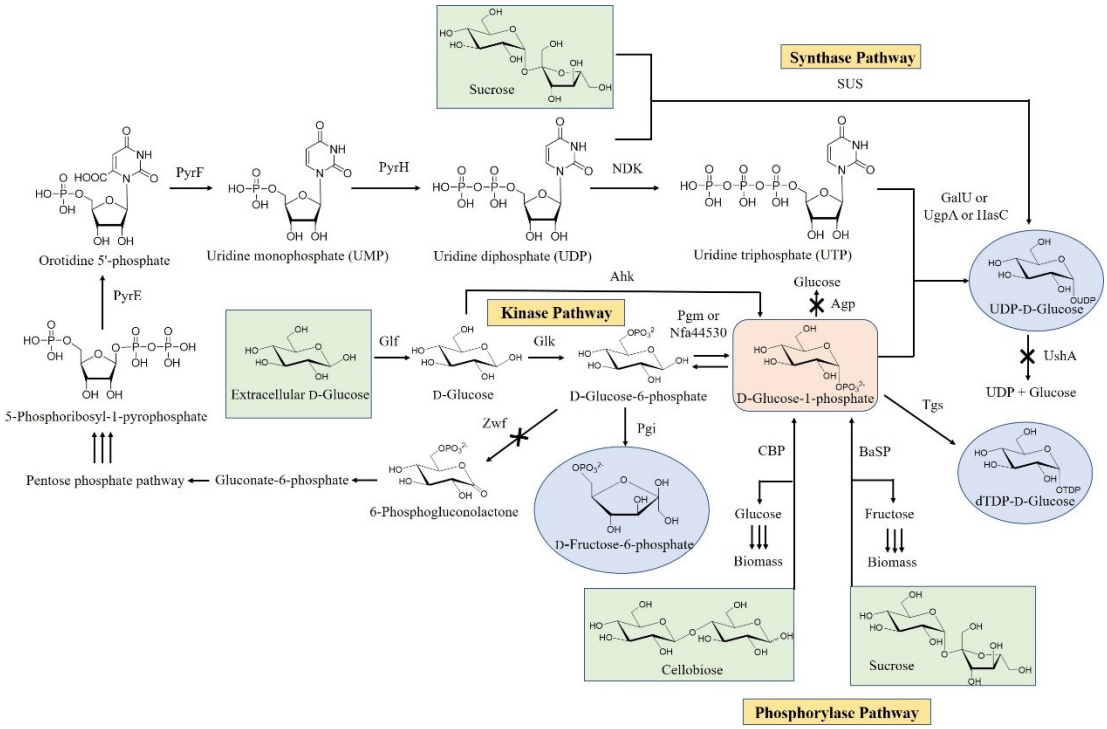

Sucrose
Synthase Pathway
SUS
Orotidine 5'-phosphate
PyrF
Uridine monophosphate (UMP)
PyrH
Uridine diphosphate (UDP)
NDK
Uridine triphosphate (UTP)
GalU or
UgpA or IIasC
UDP-D-Glucose
UshA
UDP + Glucose
PyrE
5-Phosphoribosyl-1-pyrophosphate
Extracellular D-Glucose
Glf
D-Glucose
Kinase Pathway
Glk
D-Glucose-6-phosphate
Ahk
Glucose
Agp
Pgm or
Nfa44530
D-Glucose-1-phosphate
Tgs
dTDP-D-Glucose
Zwf
Pgi
CBP
BaSP
Pentose phosphate pathway
Gluconate-6-phosphate
6-Phosphogluconolactone
D-Fructose-6-phosphate
Glucose
Biomass
Fructose
Biomass
Cellobiose
Sucrose
Phosphorylase Pathway

Figure 6

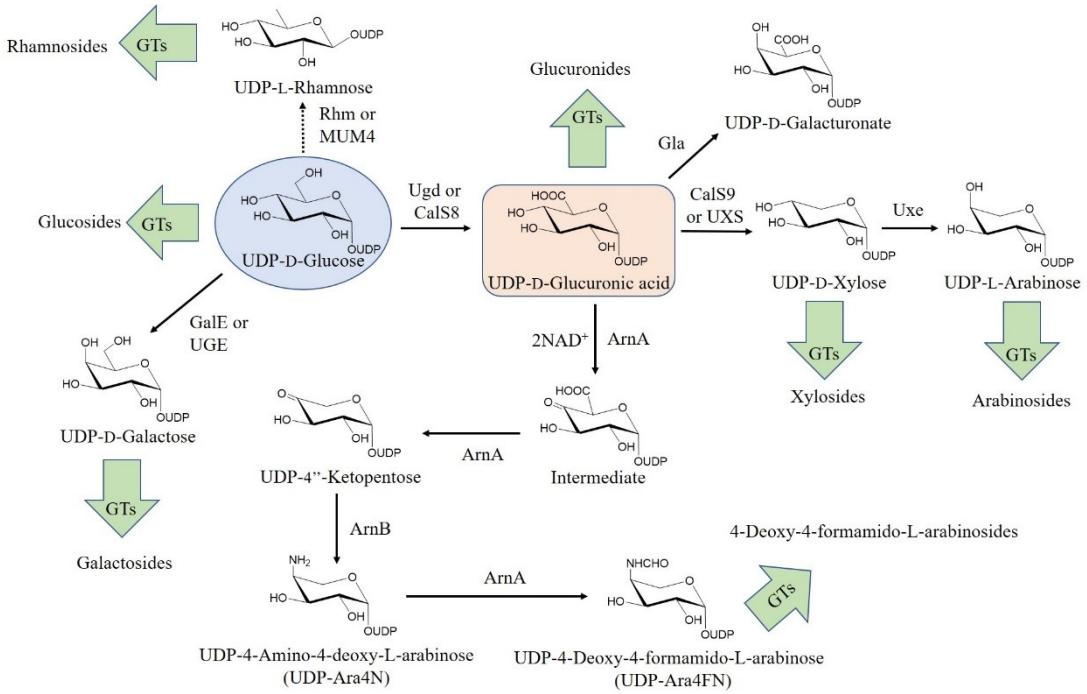

Rhamnosides
GTs
UDP-L-Rhamnose
Rhm or
MUM4
Glucosides
GTs
UDP-D-Glucose
Ugd or
CalS8
Glucuronides
GTs
UDP-D-Glucuronic acid
Gla
UDP-D-Galacturonate
CalS9
or UXS
UDP-D-Xylose
Uxe
UDP-L-Arabinose
GTs
Xylosides
GTs
Arabinosides
GalE or
UGE
UDP-D-Galactose
GTs
Galactosides
2NAD+
ArnA
Intermediate
ArnA
UDP-4''-Ketopentose
ArnB
UDP-4-Amino-4-deoxy-L-arabinose
(UDP-Ara4N)
ArnA
UDP-4-Deoxy-4-formamido-L-arabinose
(UDP-Ara4FN)
GTs
4-Deoxy-4-formamido-L-arabinosides

Figure 7

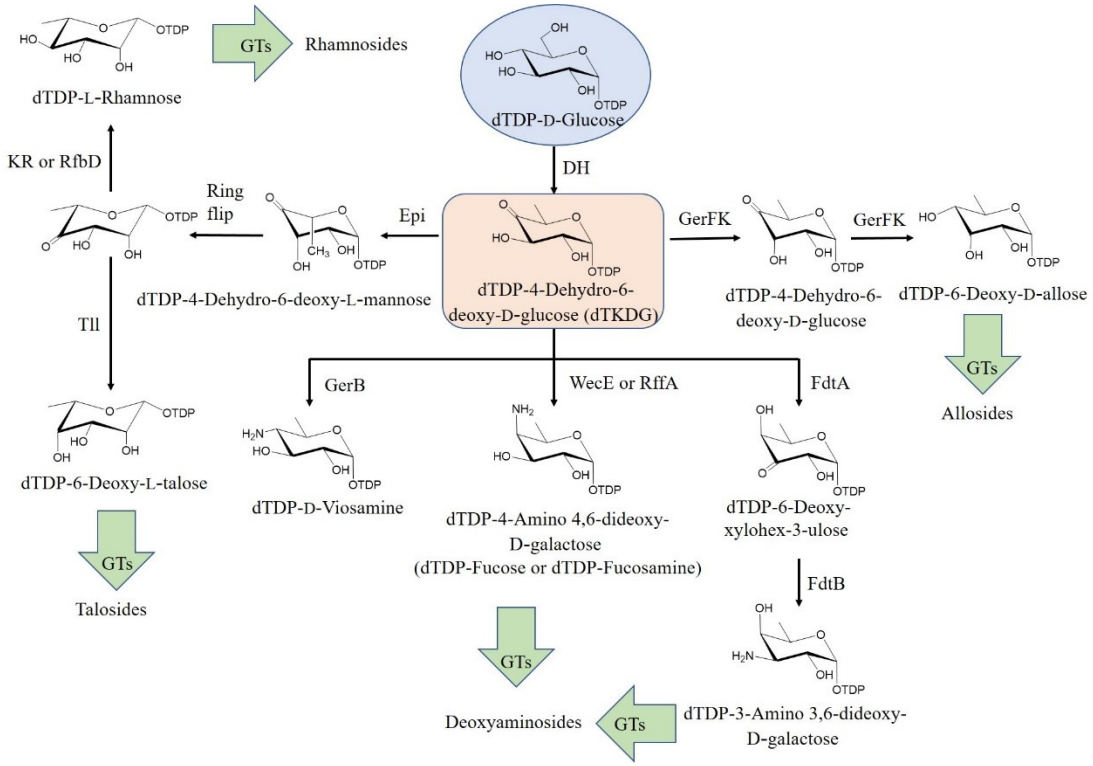

dTDP-L-Rhamnose
GTs
Rhamnosides
dTDP-D-Glucose
DH
KR or RfbD
Ring flip
Epi
GerFK
GerFK
dTDP-4-Dehydro-6-deoxy-L-mannose
dTDP-4-Dehydro-6-deoxy-D-glucose (dTKDG)
dTDP-4-Dehydro-6-deoxy-D-glucose
dTDP-6-Deoxy-D-allose
Tll
GTs
Allosides
GerB
WecE or RffA
FdtA
dTDP-6-Deoxy-L-talose
dTDP-D-Viosamine
dTDP-4-Amino 4,6-dideoxy-D-galactose (dTDP-Fucose or dTDP-Fucosamine)
dTDP-6-Deoxy-xylohex-3-ulose
FdtB
GTs
Talosides
GTs
Deoxyaminosides
GTs
dTDP-3-Amino 3,6-dideoxy-D-galactose

Figure 8

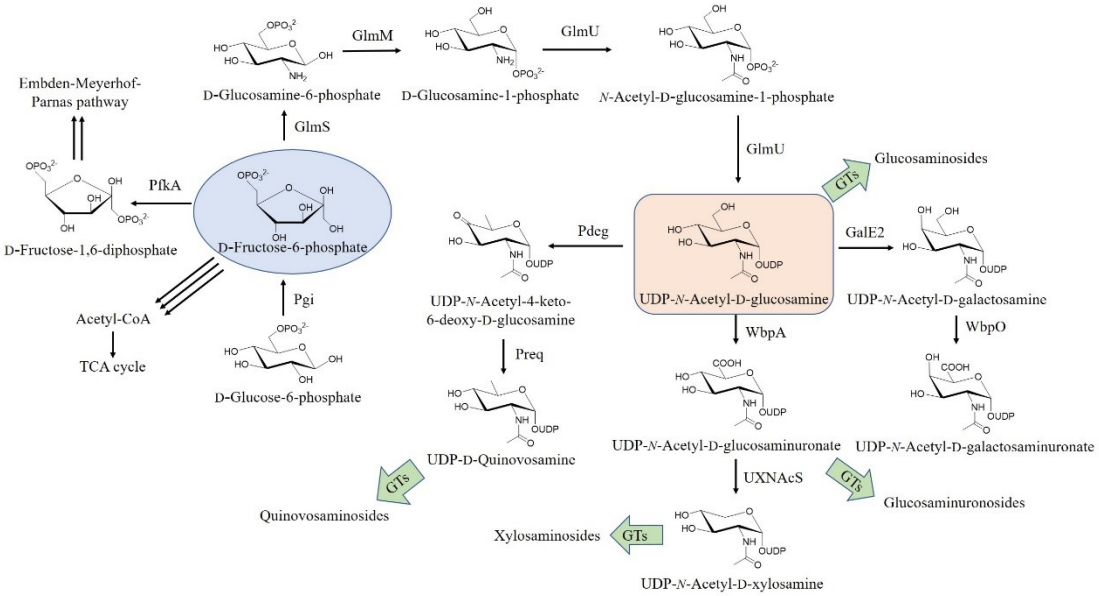
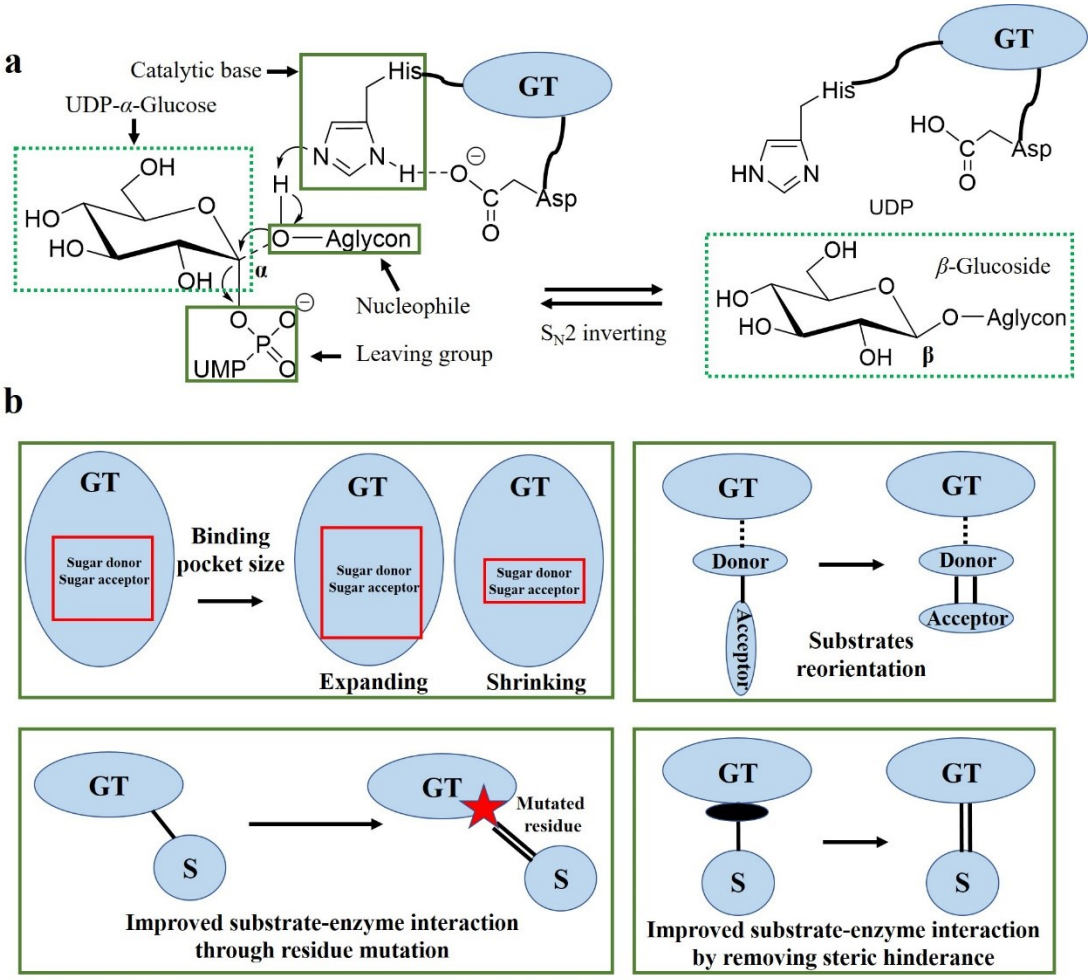

Embden-Meyerhof-
Parnas pathway
D-Glucosamine-6-phosphate
GlmM
D-Glucosamine-1-phosphate
GlmU
N-Acetyl-D-glucosamine-1-phosphate
GlmS
GlmU
Glucosaminosides
GTs
PfkA
D-Fructose-1,6-diphosphate
D-Fructose-6-phosphate
Pdeg
GalE2
UDP-N-Acetyl-D-glucosamine
UDP-N-Acetyl-D-galactosamine
UDP-N-Acetyl-4-keto-
6-deoxy-D-glucosamine
Pgi
Acetyl-CoA
TCA cycle
WbpA
WbpO
Preq
D-Glucose-6-phosphate
UDP-N-Acetyl-D-glucosaminuronate
UDP-N-Acetyl-D-galactosaminuronate
UDP-D-Quinovosamine
GTs
UXNAcS
GTs
Quinovosaminosides
Glucosaminuronosides
Xylosaminosides
GTs
UDP-N-Acetyl-D-xylosamine

Figure 9

3036

3037



3038

3039 Figure 10