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Microbial production of natural and non-natural flavonoids: Pathway engineering, directed evolution and systems/synthetic biology

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ABSTRACT

In this review, we address recent advances made in pathway engineering, directed evolution, and systems/synthetic biology approaches employed in the production and modification of flavonoids from microbial cells. The review is divided into two major parts. In the first, various metabolic engineering and system/synthetic biology approaches used for production of flavonoids and derivatives are discussed broadly. All the manipulations/engineering accomplished on the microorganisms since 2000 are described in detail along with the biosynthetic pathway enzymes, their sources, structures of the compounds, and yield of each product. In the second part of the review, post-modifications of flavonoids by four major reactions, namely glycosylations, methylations, hydroxylations and prenylations using recombinant strains are described.

Keywords: Flavonoids, pathway engineering, metabolic engineering, post-modifications, systems/synthetic biology, microbial production, biotransformation

1. Introduction

Flavonoids are natural pigments abundantly present in the plant kingdom. Structurally, they have the C₆-C₃-C₆ carbon framework. The position of linkage of B-ring to the C-ring (benzopyrano moiety) is used to sort these C-15 molecules into three different classes: flavonoids, isoflavonoids and neoflavonoids (Fig.1). Based on the degree of oxidation and saturation level in the C-ring and presence of additional rings, flavonoids are divided into eight different subclasses. Isoflavonoids also contain several different subclasses and neoflavonoids comprise only three groups. Other plant secondary metabolites containing a similar carbon skeleton, such as chalcones, stilbenes, or aurones are considered minor flavonoids. The representative structures and example of each subclass of flavonoids are presented in the Fig. 1 and Table 1.

(Fig. 1 and Table 1 around here)

1.1 Biosynthesis of flavonoids in plants

Flavonoids are biosynthesized on the cytosolic face of endoplasmic reticulum membranes by the action of various biosynthetic enzyme complexes. All these groups of compounds are derived from the phenylpropanoid pathway using three molecules of malonyl-CoA and a molecule of phenylpropanoyl-CoA. Except minor flavonoids, all others are biosynthesized via a common core unit, chalcone. Different classes of enzymes (isomerase, hydroxylase, oxido-reductase, post modification enzymes like glycosyltransferases, methyltransferases, acyltransferases) act on this core unit to diversify these molecules into the more than 8,000 known flavonoids (Veitch and Grayer, 2011; Iwashina, 2015) and approximately the 2,000 known isoflavonoids (Veitch, 2007).

The flavonoid biosynthetic pathway is one of the most studied in plants and the enzymes involved are well characterized (Winkel-Shirley, 2006; Pandey and Sohng, 2013). L-Phenylalanine or L-tyrosine produced via shikimate and arogenate pathways are the starting precursors of the phenylpropanoid pathway. Tyrosine ammonia lyase (TAL) or phenylalanine ammonia lyase (PAL) converts tyrosine or phenylalanine to their carboxylic acid derivatives, *p*-coumaric acid and cinnamic acid, respectively. The conversion of cinnamic acid to *p*-coumaric acid requires an additional enzyme, cinnamic acid 4-hydroxylase (C4H). Using one molecule of adenosine triphosphate (ATP) and one molecule of coenzyme-A (CoA), 4-coumaroyl-CoA ligase (4CL) converts *p*-coumaric acid into activated 4-coumaroyl-CoA, one of the precursors in C6-C3-C6 backbone biosynthesis of different flavonoid classes. Another precursor is malonyl-CoA, mainly produced via acetyl-CoA by the irreversible action of acetyl-CoA carboxylase (ACC) complex. Three molecules of malonyl-CoA and a molecule of 4-coumaroyl-CoA are condensed by the action of chalcone synthase (CHS) to form a C6-C3-C6 backbone unit (naringenin chalcone) in all flavonoids. Naringenin chalcone is converted into naringenin in a ring-closing step by chalcone isomerase (CHI), which is further modified to various sub-classes of flavonoids and isoflavonoids (Fig. 2). The same chalcone derivative is also converted to aurones by the constitutive action of various enzymes. For example, auroresidin synthase catalyzes the hydroxylation and/or oxidative cyclization of chalcones to aurones (Nakayama et al., 2000, 2001; Ono et al., 2006) and glucosyltransferases further modify aurones to their respective glucosides (Ono et al., 2006). In contrast, stilbenoids are synthesized by using 4-coumaroyl-CoA and malonyl-CoA in a single step condensation reaction catalyzed by stilbene synthase (STS) and circumvents the chalcone intermediate. All these aglycone molecules are further diversified by various post-modification enzymes such

as methyltransferases, glycosyltransferases, hydroxylases, acyltransferases etc (Cao et al., 2014).

(Fig. 2 around here)

1.2 Significance of flavonoids in human health

In plants, flavonoids act against various biotic and abiotic stresses such as ultraviolet radiation, microbial infections, and physical and radical damage (Winkel-Shirley, 2002; Treutter, 2006). In humans, flavonoids enter the body through consumption of plant-derived foods and play crucial roles in preventing the onset of various diseases (Williams et al., 2004). For example, flavonoids scavenge free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated into the body and inhibit damage and propagation through metal chelation (Heim et al., 2002). Free radical species cause cardiovascular diseases and neurodegenerative diseases like Parkinson's and Alzheimer's diseases. Moreover, studies found that flavonoids also play roles in the prevention and onset of cancers and diabetes (Ding et al., 2013; Xiao and Shao, 2013; Xiao and Tundis, 2013).

Some of the specific biological functions of widely studied flavonoids and their therapeutic effects are summarized in Table 2. Recently, flavonoids having antibacterial activities are attracting attention for the development of novel drugs or the adjuncts (Ozçelik et al., 2006; Ozçelik et al., 2008; Orhan et al., 2010; Pistelli and Giorgi, 2012; Kumar and Pandey, 2013; Moghaddam et al., 2014). As a result, many researches are focused on production of natural as well as novel flavonoid derivatives using recombinant microorganisms by following various metabolic and protein engineering and synthetic biology approaches. These advances will be discussed in this article.

(Table 2 around here)

2. Metabolic engineering of microbes for flavonoids production

From long ago, humans have used microorganisms to supply foods, beverages, drugs and chemicals. But recently, development of metabolic engineering tools including genome sequencing and synthesis, protein engineering, computational system biology, and synthetic biology tools have enabled biotechnologists to generate robust microbial cell factories to produce wide range of natural and non-natural primary and secondary metabolites, commodity chemicals and biofuels (Ajikumar et al., 2010; Choi and Lee, 2013; Heider et al., 2014; George et al., 2015; Na et al., 2013; Rodrigues et al., 2015; Westfall et al., 2012). Such advances have been extensively reviewed (Mora-Pale et al., 2014; Trantas et al., 2015; Wang et al., 2016; Xu et al., 2013). Flavonoids are secondary plant metabolites that have been the target of metabolic engineers for 10 to 15 years. To date, a number of groups have successfully produced and modified flavonoids from microbial sources such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Streptomyces* species (Fig. 3). Although, the structural diversity of flavonoid group of compounds is very high in nature (Wang et al., 2011a), development and production of novel and biologically potent flavonoids from microbial platforms contributes to the demand of novel drug for the treatment of various pathological conditions (Bhan et al., 2013).

Because of the well-known genetics, easy manipulation of chromosome, high cell growth rate, and easy handling of *E. coli*, *S. cerevisiae*, and *S. venezuelae*, these organisms are extensively used in industry for the production of various chemicals and compounds. However, many hurdles of using microbial cells for the production of plant or microbial derived complex molecules remain to be addressed (Mora-Pale et al., 2013). For example, many plant enzymes are difficult to express in prokaryotic microbes, such as *E. coli* because they are associated

with plant cell organelles that are absent from simple prokaryotes. In such cases, lower eukaryotes, such as *S. cerevisiae*, have been employed for the production of complex secondary metabolites, such as metabolites produced in actinomycetes (Paddon et al., 2013). Flavonoids are low molecular weight compounds with a simple 15 carbon backbone. They are mainly synthesized in plants and as such, microorganisms like *E. coli* and *S. cerevisiae* do not include endogenous genes for their biosynthesis. Flavanones, such as naringenin, are the major intermediates in flavonoid biosynthesis and much research has focused on their production in engineered microbial cells (Wu et al., 2014a). For fifteen years, various approaches have been developed and used to produce flavonoids and their derivatives from microorganisms. However, excluding a few exceptional cases, the volumetric production achieved is much lower than what is necessary to make the resulting process industrially relevant and commercially viable. Herein, we discuss the recent advancements made on the biosynthesis of flavonoids using genetically tractable microorganisms by metabolic engineering approaches. Particularly, pathway engineering, use of alternative enzymes from different sources, directed evolution, systems and synthetic biology approaches used for the production and modifications of flavonoids using microbial cells (Fig. 3) are the major interest of this review.

(Fig. 3 around here)

2.1 *De novo* pathway engineering

More than a decade ago, Horinouchi and colleagues assembled three genes specific for plant flavanone production: *RrPAL* from *Rhodotorula rubra*, *ScCCL* from *S. coelicolor* A3(2), and *GeCHS* from *Glycyrrhiza echinata*. The number of T₇ promoter and ribosome binding sites (RBS) in front of each gene was varied (Hwang et al., 2003). The recombinant *E. coli* strains

containing artificial gene cluster produced two flavanones namely pinocembrin and naringenin from phenylalanine and tyrosine, respectively. Ro and Douglas expressed putative plant phenylpropanoid pathway genes *PAL*, *C4H*, and the *C4H* redox partner cytochrome P450 reductase (*CPR*) from *P. trichocarpa* x *P. deltoides* to *S. cerevisiae* to study the carbon-flux from phenylalanine to *p*-coumaric acid (Ro and Douglas, 2004). A more extended recombinant pathway was used by Koffas and colleagues that included a *C4H* from *Arabidopsis thaliana*, a *4CL* from *Petroselinum crispum*, and a *CHS* and a *CHI* from *Petunia hybrida*. By feeding with cinnamic acid, *p*-coumaric acid and caffeic acid, three different flavanones, namely pinocembrin, naringenin and eriodictyol were produced (Yan et al., 2005). Finally, Ralston et al. (2005) also introduced partial flavonoid and isoflavonoid biosynthetic pathways in *S. cerevisiae* for production of respective compounds. These studies of heterologous expression of plant phenylpropanoid pathway genes and flavonoid modification genes into *E. coli* and *S. cerevisiae* became the first platforms for the biosynthesis of various flavonoids and related compounds. Since then, heterologous biosynthesis of flavonoids using microbial cells has attracted significant interest around the globe for production in practical quantities.

Soon after the heterologous expression of phenylpropanoid pathway genes in *S. cerevisiae* and *E. coli*, *de novo* production of flavonoids using cheap carbon sources such as glucose and amino acids was explored. Watts and colleagues in 2004 constructed a recombinant *E. coli* host harboring *TAL* from *Rba. capsulatus*; *4CL* and *CHS* from *Arabidopsis thaliana* and produced 20.8 mgL⁻¹ of naringenin without supplementing any precursor in the medium (Watts et al., 2004). In another similar study, *S. cerevisiae* was engineered to produce naringenin and pinocembrin without addition of precursors in the medium. Overexpression of

PAL from *Rhodospiridium toruloides* also exhibited *TAL* activity (L-phenylalanine ($K_m=0.29$ mM) L-tyrosine ($K_m=0.18$ mM)), *At4CL1* from *A. thaliana* accepting different propanoic acids such as *p*-coumaric acid, ferulic acid, and caffeic acid as substrates, and *CHS* from *Hypericum androsaemum* in phenylacrylic acid decarboxylase encoding *pad1* gene (decarboxylates *trans*-cinnamic acid and *p*-coumaric acid to their corresponding vinyl derivatives) knocked out strain of *S. cerevisiae*/ Δ *pad1* produced 7 mgL⁻¹ of naringenin and 0.8 mgL⁻¹ of pinocembrin. Negligible amounts of 2',4',6'-trihydroxydihydrochalcone and phloretin were also detected as by products (Jiang et al., 2005). The production titer of both naringenin and pinocembrin was enhanced to approximately 60 mgL⁻¹ in *E. coli* by expressing malonyl-CoA pool enhancing genes and supplementing amino acid precursors in the medium. Additional introduction of acetyl-CoA carboxylase (ACC) subunit genes (*accBC* and *dtbR1*) from *Corynebacterium glutamicum* in *E. coli* expressing the same set of flavonoid biosynthetic pathway genes used by Hwang et al., 2003 and addition of *Pueraria lobata* (*PLCHI*) produced 57 mgL⁻¹ of naringenin and 58 mgL⁻¹ of pinocembrin in the presence of 3 mM of tyrosine and phenylalanine in the medium (Miyahisa et al., 2005). In an effort to expand the repertoire of flavonoid molecules produced, Leonard *et al.* reported the construction of *S.cerevisiae* recombinant strains for the production of plant specific flavone molecules. Specifically, the authors introduced a soluble flavone synthase I (*PcFSI*) from parsley and an insoluble (membrane bound P450 monooxygenase) flavone synthase II (*AFNS2*) from snapdragon into the previously constructed, flavanone producing *S. cerevisiae* recombinant strain. The resulting recombinant strains were able to convert various phenylpropanoid acid precursors into the flavanone molecules chrysin, apigenin and luteolin. Improvement of flavone biosynthesis was achieved by overexpressing the yeast P450

reductase *CPR1* in the *AFNS2*-expressing recombinant strain; however, the recombinant yeast strain expressing the soluble *PcFSI* was the one that demonstrated the highest production of flavones (Fowler and Koffas, 2009; Leonard et al., 2005), with final titers in the order of 2-3 mgL⁻¹ of apigenin and luteolin. The same group also tested the production of the same flavone molecules in recombinant *E.coli* that produces flavanones. Without any strain optimization, they demonstrated the production of the flavones apigenin at 0.4 mgL⁻¹, luteolin at 10 µg/l and genkwanin at 0.2 mgL⁻¹ (Leonard et al., 2006). In a similar work in *E.coli*, the Horinouchi group also expanded the naringenin and pinocembrin producing systems to flavones and flavonols by expressing flavone synthase 1 (*FSI*) from *Petroselinum crispum* for flavones production and additional two genes flavanone 3β-hydroxylase (*F3H*) and flavonol synthase (*FLS*) from *Citrus* species for flavonols production. These flavone and flavonol producing strains produced 13 mgL⁻¹ of apigenin and 9.4 mgL⁻¹ of chrysin as flavones; 15.1 mgL⁻¹ of kaempferol and 1.1 mgL⁻¹ of galangin as flavonols from tyrosine and phenylalanine, respectively (Miyahisa et al., 2006). Flavonol production in recombinant *E.coli* was also demonstrated by Leonard *et al* through the functional expression of a plant P450 flavonoid 3',5'-hydroxylase (F3'5'H) as a fusion protein with a plant P450 reductase. By creating such a translational fusion, the authors mimicked the protein structure of the soluble bacterial P450s, such as the soluble BM3 P450 found in *Bacillus megaterium*. This allowed the production of the flavonols kaempferol and quercetin, albeit at small final titers (Leonard et al., 2006). In another study, in an effort to produce isoflavones in *E.coli*, the same team of investigators created a series of isoflavone synthases (the P450 monooxygenases that catalyze the conversion of precursor flavanones to isoflavones) that were truncated at their N-terminus which was replaced by tailor-made membrane recognition signals. The specific, in vivo

production of isoflavones (specifically genistein) catalyzed by one of these artificial chimeras was up to 20-fold higher than that achieved by the native isoflavone synthase expressed in the eukaryote *S. cerevisiae* and up to 10-fold higher than the production achieved by soy beans (Leonard and Koffas, 2007). In another study, four metabolically engineered *S. cerevisiae* strains were generated by assembling flavonoid biosynthetic pathway genes from different sources. *PAL* and *CPR* from *Populus* hybrid (*P. trichocarpa* x *P. deltoids*), *C4H*, *4CL*, *CHS*, *CHI*, isoflavone synthase (*IFS*), flavanone 3-hydroxylase (*F3H*), and flavonol 3'-hydroxylase (*F3'H*) from *Glycine max*, resveratrol synthase (*RS*) from *Vitis vinifera* cv. Soultanina, and *FLS* from *Symphytum tuberosum* were assembled in various combinations for production of stilbenes, flavanones, isoflavones, and flavonols. Each engineered strain was specific for production of a single compound from phenylalanine. The engineered strains produced 0.29 mgL⁻¹ of *trans*-resveratrol, 8.9 mgL⁻¹ of naringenin, 0.1 mgL⁻¹ of genistein, 0.9 mgL⁻¹ of kaempferol and 0.26 mgL⁻¹ of quercetin under optimal growth conditions while supplementing 10 mM of phenylalanine in the medium. The production of the same compounds was higher (Table-3) upon supplementation of intermediates such as *p*-coumaric acid and naringenin (Trantas et al., 2009).

In plants, the different isoforms of enzymes are hypothesized to be organized in one or more enzyme complexes to promote substrate channeling. Koopman and colleagues studied a subset of isoenzymes from *A. thaliana* by BAR Expression Angler (Toufighi et al., 2005) for expression profile correlation analysis. Among several sets of genes included in the expression correlation studies, five flavonoid biosynthetic genes *At4CL3*, *CHS1*, *CHI1*, codon optimized *C4H* and *PAL1*, and two codon optimized *CPR* variants (*CPR1* or *CPR2*) were used for naringenin biosynthesis in *S. cerevisiae*. The best assembled strain produced 1.47

mgL⁻¹ of extracellular naringenin in shake-flask cultures containing synthetic medium and glucose as the sole carbon source. Engineering of strain by introducing tyrosine insensitive 3-deoxy-D-arabinose-heptulosonate-7-phosphate (DAHP) synthase *ARO4* allele (*ARO4*^{G226S}), knock-out of *ARO3*, and phenylpyruvate decarboxylase genes (*PDC1*, *PDC5* and *PDC6*) enhanced the production of naringenin to 12.6 mgL⁻¹ along with production of 200 µM of coumaric acid and phloretic acid as side products. Both byproducts were converted into naringenin by overexpression of additional copies of a codon optimized *CHS3* and a yeast codon optimized *TAL1* gene with known high catalytic efficiency from *R. capsulatus*, resulting in 54.45 mgL⁻¹ of naringenin. Under controlled conditions in a 2-l fermenter, the strain produced 112.9 mgL⁻¹ of naringenin without addition of any precursor in the medium (Koopman et al., 2012). *De novo* biosynthesis of other flavonoids from simple precursors includes anthocyanins (cyanidin 3-*O*-glucoside and pelargonidin 3-*O*-glucoside), flavan-3-ols ((+)-catechin and afzelechin), 5-deoxyflavanones (liquiritigenin), eriodictyol and resveratrol derivatives. Anthocyanins were produced by cloning a four step metabolic pathway that allowed the conversion of flavanone precursors into their corresponding anthocyanin molecules. The pathway included a flavanone 3-hydroxylase (*F3H*) from apple (*Malus domestica*) a dihydroflavonol reductase (*DFR*) from *Anthurium andraeanum*, an anthocyanidin synthase (*ANS*) also from apple and an anthocyanin 3-*O*-glucosyltransferase (*3-GT*) from *Petunia hybrida*. The selection of *DFR* and *ANS* was based on screening various plant enzymes from different plant sources recombinantly expressed in *E.coli* (Leonard et al., 2008). Two different anthocyanins, the purple cyanidin 3-*O*-glucoside and the orange pelargonidin 3-*O*-glucoside were produced by feeding the recombinant *E.coli* strain with their corresponding precursor flavanones, eriodictyol and naringenin, respectively, albeit at

extremely low quantities of only a few micrograms per liter. Despite the low production, this was the first demonstration of the production of the unstable anthocyanins by a recombinant host and one of the first publications on recombinant production of flavonoid molecules (Yan et al., 2005). In another paper, Chemler *et al.* presented the production of flavan-3-ols, such as green tea catechins, in recombinant *E.coli* first from their flavanone precursors and then from their phenylpropanoic acid precursors. This was accomplished by constructing a recombinant pathway that included F3H from apple, DFR from *A. andraeanum* and leucoanthocyanin reductase (LAR) from *Antirrhinum majus*. While the production levels achieved were in the order of a few milligrams per liter, this was the first demonstration of the production of some rare catechins, such as afzelechin, in a recombinant host (Chemler et al., 2007). This production level was later increased to almost one gram per liter after extensive pathway engineering described later in this paper.

In addition to the common flavanones, leguminous plants have evolved a distinct class of flavanone molecules known as 5-deoxyflavanones. Yan *et al.* constructed a four-step metabolic pathway in *E. coli* with *4CL* derived from parsley, *CHS* from petunia and *CHI* and chalcone reductase (*CHR*) from the leguminous plant *Medicago sativa*. By optimizing feeding and fermentation strategies, the authors were able to reach a production of 17 mgL⁻¹ of liquiritenin and 3.5 mgL⁻¹ of isoliquiritenin in the constructed recombinant strain. By feeding with cinnamic and caffeic acids, other deoxyflavanones were also produced including 7-hydroxyflavanone (1.9 mgL⁻¹), butin (4.2 mgL⁻¹) and butein (0.5 mgL⁻¹). The same pathway was also introduced in *S. cerevisiae*; however, the volumetric titers of the 5-deoxyflavanones produced were lower than the production achieved in *E. coli* (Yan et al., 2007). In a later study, eriodictyol was produced from L-tyrosine in *E. coli* recombinant strain.

R. glutinis originated *TAL*, *P. crispum* originated *4CL*, *CHS* from *P. X hybrida*, and *CHI* from *M. sativa* were used along with ACC complex genes (*accBC*, *dtsR1* from *C. glutamicum*) and *acs* from *E. coli* to produce naringenin. Naringenin was further modified by hydroxylation using a fusion protein of truncated *F3'H* from *Gerbera hybrida* and *CPR* from *Catharanthus roseus* to eriodictyol. Upon modification of the host strain by deletion of acetate kinase (*ackA*) the resulting strain produced 5.70 mgL⁻¹ of eriodictyol from glucose. The production titer reached 107 mgL⁻¹ when sufficient L-tyrosine was exogenously supplemented and ACC complex genes were overexpressed in a high copy number plasmid (Zhu et al., 2014). Similarly, in order to produce various precursors of flavonoids from simple carbon sources, Hong and his group constructed *E. coli* strain producing high titers of tyrosine of about 400 mgL⁻¹. The strain was further engineered by incorporating plasmids harboring a codon optimized *TAL* and 4-coumarate 3-hydroxylase (*C3H/sam5*) from *Saccharothrix espanaensis*, and an *O*-methyltransferase (*COM*) gene from *A. thaliana* assembled in a monocistronic fashion with an individual promoter, ribosome binding site, and terminator for each gene. The strain produced 974 mgL⁻¹, 150 mgL⁻¹, and 196 mgL⁻¹ of *p*-coumaric acid, caffeic acid, and ferulic acid, respectively, from glucose (Kang et al., 2012). The same group extended the pathway for methylated resveratrol production and assembled codon optimized *S. espanaensis* *TAL*, two *O*-methyltransferase (*sbOMT1* and *sbOMT3*) genes from *Sorghum bicolor*, and a codon optimized *STS* from *Arachis hypogaea* (*AhSTS*), and *ScCCL* from *S. coelicolor* A3(2) in a monocistronic fashion under T₇ promoter. The recombinant *E. coli* produced resveratrol along with three methylated resveratrol derivatives (3,5-dihydroxy-4'-methoxystilbene (~2.5 mgL⁻¹), 3,4'-dimethoxy-5-hydroxystilbene (~0.17 mgL⁻¹), and 3,5,4'-trimethoxy stilbene (~0.05 mgL⁻¹)) from simple sugar (Kang et al., 2014). Moreover,

introduction of an additional glycosyltransferase gene (*yjiC*) from *Bacillus licheniformis* DSM 13 (Pandey et al., 2013a; 2014a,b,c) to the resveratrol biosynthesis monocistronic pathway produced resveratrol-4'-*O*-glucoside (7.5 mgL⁻¹), *trans*-resveratrol-3-*O*-glucoside (*trans*-piceid) (2.5 mgL⁻¹), and *cis*-resveratrol-3-*O*-glucoside (1.7 mgL⁻¹) from glucose (Choi et al., 2014).

Other examples of *de novo* biosynthesis of flavonoid derivatives from *E. coli* include methylated flavanones (sakuranetin, ponciretin) and kaempferol 3-*O*-rhamnoside (Fig. 4). Ahn and his group achieved successful production of flavonoid derivatives by engineering *E. coli*. To produce methylated flavanones from glucose, *E. coli* strain was mutated by disrupting three chromosomal genes (*tyrR* encoding for phenylalanine DNA-binding transcription repressor, *pheA* encoding prephenate dehydratase/chorismate mutase, and *icdA* encoding for isocitrate dehydrogenase) to divert carbon-flux towards tyrosine and CoA, two initial precursors of flavonoids. *E. coli*/Δ*tyrR*Δ*pheA*Δ*icdA* was further engineered by overexpressing genes encoding for phosphoenolpyruvate (*ppsA*), transketolase (*tktA*), feedback inhibitor insensitive deoxyphosphoheptone aldolase (*aroG^{fbr}*) and prephenate dehydrogenase (*tyrA^{fbr}*) from *E. coli* along with heterologous flavonoid biosynthesis genes from different sources which included *S. espanaensis* (*TAL*), *O. sativa* (*4CL*), *Populus euramericana* (*CHS*), *G. max* (*SOMT2*) or *O. sativa* (*NOMT*). The engineered strains produced 42.5 mgL⁻¹ of ponciretin and 40.1 mgL⁻¹ of sakuranetin, respectively (Kim et al., 2013a). The same background *E. coli* strain overexpressing same precursors enhancing genes and flavonoid biosynthesis genes (additional *FLS* from *P. euramericana* and *AtUGT78D1* from *A. thaliana* in place of *OMTs*) produced 57 mgL⁻¹ of kaempferol 3-*O*-rhamnoside (Yang et al., 2014).

2.2 Central carbon flux redirection

The supply of malonyl-CoA is considered as one of the major bottlenecks of biosynthesis of flavonoids. This precursor is required in the condensation reaction catalyzed by CHS, where three molecules of cytosolic malonyl-CoA are sequentially added to one molecule of phenylpropanoyl-CoA. Moreover, it is also one of the important precursors for biosynthesis of most polyketides and fatty acids. Thus, several efforts have been carried out to increase the pool of malonyl-CoA in microbial cytosol for the production of important polyketides, flavonoids, and long chain fatty acids. Malonyl-CoA pool availability has been enhanced in the cell by overexpression of ACC complex genes (Leonard et al., 2007; Wattanachaisaereekul et al., 2008) and knockout of *ackA* and acetaldehyde dehydrogenase (*adhE*) (Zha et al., 2009) (Fig. 4). However, system biology approaches have been recently applied to design improved biocatalysts by analyzing genome-scale metabolic networks to identify minimal potential target genes for the enhanced production of malonyl-CoA and target secondary metabolites (Lim et al., 2011; Bhan et al., 2013; Xu et al., 2011). This section discusses various strategies used for enhancement of malonyl-CoA and other cofactors pool necessary for the production of flavonoids and derivatives.

(Fig. 4 around here)

Malonyl-CoA biosynthesis genes, acetate assimilation genes, and malonate assimilation genes are mostly overexpressed to enhance the malonyl-CoA pool and thus flavonoids in many studies. Moreover, the malonyl-CoA and its precursors consuming pathway genes are also engineered to dam up the flow of malonyl-CoA and its precursors to other diverting pathways. Alternatively, orotic acid assimilation pathway and central uridine diphosphate

(UDP)-glucose biosynthetic pathways are also engineered to enhance the biosynthesis of UDP-glucose, the limiting cofactor in the biosynthesis of flavonoid glycosides (Fig. 4). For example, ACC complex encoding genes (*accABCD*) along with biotin ligase (*BirA*) from *Photorhabdus luminescens* were expressed with acetate assimilation pathway genes (*ackA*, phosphotransacetylase (*pta*) and acetyl-CoA synthase (*acs*)) of *E. coli*. The engineered strain produced 429 mgL⁻¹ of pinocembrin, 119 mgL⁻¹ of naringenin, and 52 mgL⁻¹ of eriodictyol which is 1,379%, 183%, and 373% higher in comparison to the control strain overexpressing only flavonoid biosynthesis genes (*Pc4CL2* from *P. crispum*, *PhCHS* from *Petunia x hybrida* and *MsCHI* from *Medicago sativa*) supplemented with cinnamic acid, *p*-coumaric acid and caffeic acid, respectively (Leonard et al., 2007). In order to improve the biosynthesis of flavanones and anthocyanins, alternative carbon assimilation pathways were used along with plant biosynthetic genes and inhibition of competitive reaction pathways using chemical inhibitors. Instead of overexpression of ACC complex for conversion of glucose to malonyl-CoA via acetyl-CoA, *matB* and *matC* from *Rhizobium trifolii* along with *Pc4CL2*, *PhCHS*, and *MsCHI* were used for the production of various flavanones from exogenously added phenylpropanoic acids, glucose, and malonate. The introduction of malonate assimilation pathway increased by 250% the biosynthesis of natural flavanone. The exogenous supplement of malonate, a direct source of endogenous malonyl-CoA and overexpression of malonate assimilation genes allowed the generation of malonyl-CoA bypassing central carbon metabolism. The malonyl-CoA in cell is primarily utilized in fatty acid metabolism. Thus, to circumvent the depletion of malonyl-CoA in the cell, fatty acid biosynthesis pathway enzymes (*FabB* and *FabF*) were inhibited by addition of cerulenin in the culture medium which resulted in production of 710 mgL⁻¹ of pinocembrin, 186 mgL⁻¹ of naringenin, and 54

mgL⁻¹ of eriodictyol which is more than 900% enhancement in production in comparison to control *E. coli* strain expressing only flavonoid biosynthetic pathway genes. High dose of cerulenin decreased production of flavonoids and also reduced the cell growth rate possibly due to the detrimental effect of malonyl-CoA on cell growth (Subrahmanyam et al., 1998). Thus, systematic enhancement of malonyl-CoA precursor is vital for increased production of flavonoids in *E. coli*. On the other hand, orotic acid assimilation pathway was also overexpressed for efficient biosynthesis of cyanidin 3-*O*-glucoside, an anthocyanin whose biosynthesis requires a glycosyltransferase that transfers the glucose moiety from UDP-glucose to the aglycone flavylum cation (anthocyanidin). Intracellular availability of UDP-glucose has been shown to be the limiting factor in the biosynthesis of anthocyanins in recombinant *E. coli*. Hence, to produce various anthocyanins from flavan-3-ol precursors (afzelechin, (+)-catechin), anthocyanin synthase (*ANS*) from *P. hybrida* and 3-*O*-glucosyltransferase (*3-GT*) from *A. thaliana* were overexpressed along with orotic acid assimilation genes (*pyrE*, *pyrF*, *cmk*, *ndk*). In addition, the *E. coli* glucose-1-phosphate biosynthetic gene phospho-glucomutase (*pgm*) and the *E. coli* glucose-1-phosphate uridylyltransferase (*galU*) that are required for the synthesis of UDP-glucose were also overexpressed while the UDP-glucose utilizing pathway genes *udg*, *galE* and *galT* were deleted. The best strain produced up to 104 mgL⁻¹ of cyanidin 3-*O*-glucoside and 113 mgL⁻¹ of pelargonidin 3-*O*-glucoside from exogenous (+)-catechin and afzelechin, respectively (Leonard et al., 2008).

2.3 Alternative enzymes selection

Isoenzymes from different sources exhibit altered catalytic properties and therefore, proper selection of a gene from appropriate sources is a prerequisite for optimization of biosynthetic

pathways for heterologous production of secondary metabolites. Since the flavonoid biosynthesis pathway is well studied in different plants, a number of phenylpropanoid pathway genes and flavonoid synthesis genes have been functionally characterized from different sources. Thus, studies have also been carried out where alternative flavonoid biosynthetic pathway genes have been assembled from different sources with the goal of higher production.

Koffas and his group in 2005 investigated the use of two different flavone synthase enzymes *FSI* (soluble) from *P. crispum* and *FSII* (membrane bound) from *Antirrhinum majus* cv. Montego Yellow for the production of flavones in *S. cerevisiae*. Previously characterized flavonoid biosynthetic genes *C4H* of *A. thaliana*, *Pc4CL-2* from *P. crispum*, *PhCHS* and *PhCHI* from *P. hybrida* along with *FSI* or *FSII* harboring recombinant strains were generated. Functional *FSII* enzyme requires NADPH and is inhibited by Fe^{2+} while *FSI* requires 2-oxoglutarate, Fe^{2+} , and ascorbate as cofactors. Supplementation of *p*-coumaric acid in the recombinant strains resulted in production of chrysin, apigenin, and luteolin as well as several of their precursors. However, comparative analysis showed 50% more apigenin production from *FSI*-expressing recombinant strain than *FSII*-expressing recombinant strain (Leonard et al., 2005). Similarly, *PhCHI* was found to be rate limiting for biosynthesis of flavanones. Replacement of *PhCHI* by *MsCHI* from *M. sativa* resulted in enhanced production of pinocembrin, naringenin and eridictyol. In order to circumvent feedback inhibition issues, the *BirA* from *P. luminescens* instead of the *E. coli* *BirA* was overexpressed in order to enhance the biotinylation of acetyl-CoA carboxylase (Leonard et al., 2007). In an effort to improve anthocyanin production, the same group reconstructed anthocyanin biosynthetic pathways in *E. coli* using genes from various plants and also diverted central carbon fluxes towards UDP-

glucose. One of the key enzymatic steps in anthocyanin biosynthesis is the 2-oxoglutarate-dependent dioxygenase activity of *ANS* which utilizes ferrous iron and sodium ascorbate as cofactors. Thus, activity of *ANS* from different plant species was studied prior to producing anthocyanin from various precursors in *E. coli*. *ANS* from *A. majus* (*AmANS*), *G. hybrida* (*GhANS*), *P. hybrida* (*PhANS*), and *Malus domestica* (*MdANS*) were introduced in combination with *A. thaliana* GT (*At3GT*) to study the production of cyanidin 3-*O*-glucoside from exogenously added (+)-catechin, UDP-glucose and other necessary co-factors such as ammonium iron (II) sulfate and sodium ascorbate in the medium. Use of *PhANS* resulted in higher production of anthocyanin (3.21 mgL⁻¹) than other *ANS*s tested. The engineered *E. coli* strain harboring *MdF3H* from *M. domestica*, dihydroflavonol reductase (*DFR*) from *A. andraeanum*, *PhANS*, *At3GT* from *A. thaliana*, and leucoanthocyanidin reductase (*DuLAR*) from *Desmodium uncinatum* produced 0.98±0.09 mgL⁻¹ of pelargonidin 3-*O*-glucoside and 2.07±0.42 mgL⁻¹ of cyanidin 3-*O*-glucoside from naringenin and eridictyol, respectively. When an additional gene, *Dv3MaT*, encoding for anthocyanidin 3-*O*-glucoside-6''-*O*-malonyltransferase from *Dahlia variabilis* was introduced, pelargonidin 3-*O*-6''-*O*-malonylglucoside (0.10 mgL⁻¹) and cyanidin 3-*O*-6''-*O*-malonylglucoside (0.12 mgL⁻¹) were produced from naringenin and eriodictyol, respectively. Later, enhanced production of anthocyanins was achieved by using flavan-3-ols as initial precursors, by creating a translational fusion between *PhANS* and *At3GT*, by engineering UDP-glucose pathway (overexpression of *pgm* and *galU*) in *E. coli*, and by optimizing fermentation conditions such as pH, that greatly affect the stability of anthocyanins. The final titer of cyanidin 3-*O*-glucoside was 70.7 mgL⁻¹ and pelargonidin 3-*O*-glucoside was 78.9 mgL⁻¹ while feeding respective flavan-3-ol precursors (+)-catechin and afzelechin (Yan et al., 2008).

Similarly, for production of resveratrol from *p*-coumaric acid in *E. coli*, resveratrol biosynthesis pathway genes *4CL* and *STS* from various sources were assembled in different combinations. Two *4CLs* (*At4CL1* and *Pc4CL2*) and eight *STSs* from seven different sources (*Pinus strobus* (*PsSTS*), *P. massoniana* (*PmSTS*), *P. densiflora* (*PdSTS*), *Psilotum nudum* (*PnSTS*), *Polygonum cuspidatum* *STSs* (*Pcu1STS* and *Pcu3STS*), *V. vinifera* (*VvSTS*) and *A. hypogaea* (*AhSTS*)) were used for the construction of recombinant *E. coli* strains. All strains were compared for the production of resveratrol under two different promoters (T7 and P_{GAP}) in two different background strains (*E. coli* BL21 star and *E. coli* BW27784). The combination of *At4CL1* with *VvSTS* was found to result in the best resveratrol production when expressed in *E. coli* BW27784 strain in conjunction with overexpression of *ACC/BirA* and in the presence of cerulenin, a specific inhibitor of *fabB-fabF* gene products. The strain produced 2,340±90 mgL⁻¹ of resveratrol while feeding *p*-coumaric acid. The production of resveratrol expressing other *STSs* was significantly lower in comparison to *VvSTS* (Lim et al., 2011).

Stephanopoulos and his group also found that the use of enzymes derived from different sources is crucial for high level production of naringenin from glucose. *E. coli* recombinant strain exhibiting 90-fold to 160-fold higher catalytic efficiency for L-tyrosine over L-phenylalanine and harboring a *Rhodobacter sphaeroides* *TAL* (*RsTAL/hutH*), a *S. coelicolor* *4CL-2* (*ScCCL-2*), an *A. thaliana* derived *CHS* (*AtCHS*), and a *P. lobata* originated codon-optimized *PICHI* genes could not produce detectable amount of naringenin even when 500 mgL⁻¹ of L-tyrosine was supplemented in the medium. Alternative selection of *PhCHS* from *P. hybrida* and *MsCHI* from *M. sativa* in place of *AtCHS* and *PICHI* achieved 6 mgL⁻¹ production of naringenin. The production further increased to 9 mgL⁻¹ upon expression of

both genes under constitutive promoter (P_{GAP}). Moreover, the combination of *PhCHS* and *MsCHI* with Ptrc-driven *RgTAL* derived from *R. glutinis* and *P. crispus* derived from *Pc4CL-1* led to the production of 29 mgL^{-1} of naringenin. The overexpression of malonate assimilation pathway genes *matB* and *matC* from *R. trifoli* and supplementation of cerulenin resulted in naringenin production of 84 mgL^{-1} (Santos et al., 2011). In another study, the enzyme combinations from different sources were examined for production of catechins from flavanones. Biosynthesis of catechins from naringenin/eriodictyol requires three enzymes: *F3H* a 2-oxoglutarate dependent dioxygenase that utilizes Fe^{2+} and ascorbate as co-factors, and *DFR* and *LAR* that utilize NADPH as an electron donor. Three *F3H*- encoding genes (*CsF3H* from *Camellia sinensis*, *MdF3H* from *M. domestica* and *PcF3H* from *P. crispum*), three *DFR* genes (*AaDFR* from *Anthurium andraeanum*, *CsDFR* from *C. sinensis*, and *FaDFR* from *Fragaria ananassa*), and two *LAR* genes (*CsLAR* from *C. sinensis* and *DuLAR* from *D. uncinatum*) of different origins were codon optimized, modified, and synthesized to construct eighteen different pathway variants using the ePathBrick vectors (Xu et al., 2012; Xu and Koffas, 2013). Among the 18 different combinations constructed, the recombinant strain expressing *CsF3H-CsDFR-DuLAR* exhibited maximum production of (+)-catechin ($65.1 \pm 0.9 \text{ mgL}^{-1}$) from 167 mgL^{-1} of exogenously added eriodictyol which was a 156% increase in production in comparison to previous report (Chemler et al., 2007). However, maximum (+)-afzelechin ($39.5 \pm 0.9 \text{ mgL}^{-1}$) was produced by the *CsF3H-FaDFR-DuLAR* combination which was very close to the titer ($38.9 \pm 1.6 \text{ mgL}^{-1}$) produced by *CsF3H-AaDFR-DuLAR* combination, revealing the significance of selection of alternative enzymes for the production of target compounds. The results also showed enhanced production of (+)-catechin ($374.6 \pm 43.6 \text{ mgL}^{-1}$) upon expression of two additional copies of each *CsDFR* and

DuLAR in *CsF3H-CsDFR-DuLAR* harboring plasmid. Since DFR and LAR require NADPH as a cofactor, an *E. coli* strain deficient in glucose-6-phosphate isomerase encoding *pgi* and phosphoenolpyruvate carboxylase encoding *ppc* genes which improve the intracellular NADPH pool enhanced (+)-catechin production to $760.9 \pm 84.3 \text{ mgL}^{-1}$; however, this titer was achieved by using the *CsF3H-AaDFR-DuLAR* gene combination. Finally, by using a library of scaffolding proteins for pathway optimization, 910.9 mgL^{-1} of (+)-catechin was obtained from 1.0 gL^{-1} of eriodictyol in *E. coli* $\text{BL}\Delta\text{pgi}\Delta\text{ppc}$ mutant (Zhao et al., 2015).

Finally, in a recent publication Jones et. al. demonstrated the use of *E.coli* co-cultures for the first time for the efficient, high-yield production of flavan-3-ols.

2.4 Protein engineering/mutagenesis

Site directed mutagenesis of proteins is usually carried out to identify the target catalytic amino acids of a protein. However, the alteration of the amino acid also generates novel enzyme mutants that can exhibit different catalytic efficiency while accepting different substrates for reaction. For example, unnatural polyketide scaffolds (Abe and Morita, 2010), alkaloid scaffolds (Morita et al., 2012), oleandomycin glycosides (Gantt et al., 2008), and commodity small molecules (Bhan et al., 2013) have been synthesized by site directed mutagenesis of different enzymes. The protein engineering and mutagenesis has also been applied for improved flavonoid production efficiency from microbes. For example, three enzymatic steps of resveratrol biosynthesis were assembled in *S. cerevisiae* by introducing *RsTAL* from *R. sphaeroides*, *At4CL* from *A. thaliana* and *VvSTS* from *V. vinifera*. *S. cerevisiae* harboring *4CL* and *STS* produced $650 \text{ }\mu\text{gL}^{-1}$ of extracellular resveratrol upon supplementation with *p*-coumaric acid. However, the translational fusion protein of *At4CL* and *VvSTS*, generated by replacing stop codon of *At4CL* by a three amino acid linker followed by *VvSTS*

open reading frame enhanced resveratrol production to 5250 μgL^{-1} . The efficient channeling of intermediates between enzymes or two active sites is found to be crucial for high titer production of the final compound, and this titer of resveratrol was 3,500-fold higher than the amount reported by Becker et al. (2003). The use of yeast preferred codon-optimized *RsTAL* in conjunction with fused *At4CL-VvSTS* radically increased *p*-coumaric acid as well as resveratrol biosynthesis. Without adding tyrosine in the medium, the recombinant yeast produced 1.06 mgL^{-1} of resveratrol after 48 h of incubation, but production increased to 1.90 mgL^{-1} when tyrosine was added (Wang et al., 2011b). Similarly, after selecting a combination of *A. thaliana At3GT* and *P. hybrida PhANS* from four different origins (*A. majus (AmANS)*, *G. hybrida (GhANS)*, *P. hybrida (PhANS)*, and *M. domestica (MdANS)*) to optimize the conversion of (+)-catechin to cyanidin, two genes *At3GT* and *PhANS* were translationally fused, creating two different types of translational fusions between *PhANS* and *At3GT* depending on the order the two genes were placed next to each other. In the first module, *PhANS* was fused to the *N*-terminus of *At3GT* while in second module the order of the genes was reversed. Both enzymes were connected via a minilinker coding for Ser-Ser-Gly-Ser-Gly. The fusion protein having *At3GT* at *N*-terminus of *PhANS* exhibited improved activity and led to approximately 17% improvement in cyanidin 3-*O*-glucoside (45.5 mgL^{-1}) production compared to tandem expression (38.9 mgL^{-1}). Co-expression of fusion protein with *galU* and *pgm* resulted in 70.7 mgL^{-1} of cyanidin 3-*O*-glucoside and 78.9 mgL^{-1} of pelargonidin 3-*O*-glucoside from (+)-catechin and (+)-afzelechin, respectively (Yan et al., 2008).

Recently, wild type *V. vinifera STS (WtVvSTS)* variants were generated by homology modelling with previously resolved crystal structure of *STS* from *A. hypogaea*. *WtVvSTS* was able to accept various CATL and BYN-type pyrones (Abe et al., 2004; Morita et al., 2001).

The specificity of *VvSTS* was further expanded by using varying sizes of starter precursors such as propionyl-CoA, myristoyl-CoA, octanoyl-CoA and methylmalonyl-CoA instead of natural malonyl-CoA. All starter precursors were accepted by *WtVvSTS* to produce various non-natural aromatic polyketides, however the mutant *VvSTSs* generated different polyketides from *WtVvSTS* using those aforementioned –CoAs coupled with malonyl-CoA as an extender unit (Bhan et al., 2015a,b). These studies expanded the possibility of production of various kinds of natural and non-natural flavonoid molecules by mutagenesis of type III polyketide synthases.

(Table 3, around here)

2.5. Precursor directed synthesis of flavonoids

In general, chemical synthesis of flavonoid compounds is often complex and expensive because of multiple hazardous synthesis steps and low final yields. Therefore, precursor directed synthesis is an attractive alternative for creating both natural and non-natural flavonoid analogues from inexpensive starting materials and endogenous precursors and cofactors (Chemler et al., 2007, 2010b; Fowler et al., 2011; Horinouchi, 2008, 2009; Mora-Pale et al., 2013).

Phenylpropanoic acids and flavanone intermediates are the major precursors of flavonoids produced from plants (Fig. 2 and Fig. 4). Therefore, these molecules are often exogenously supplemented to the engineered strains to produce different final compounds. In few experiments, unnatural flavonoid compounds have been synthesized by supplementing non-natural chemically synthesized phenylpropanoic acid precursors or flavanone derivatives. In some cases, precursor molecules that structurally do not resemble phenylpropanoic acids have also been accepted as initial precursor substrates by flavonoid biosynthetic enzymes to

produce entirely different molecules (Bhan et al., 2015a). A number of studies have been carried out to generate natural and novel flavonoid compounds by the approach discussed herein.

The four-step anthocyanin biosynthetic pathway consisting of *MdF3H* and *MdANS* from *M. domestica*, *AaDFR* from *A. andraeanum*, and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (*UGT3*) from *P. hybrida* was introduced in *E. coli* to produce two anthocyanins, pelargonidin 3-*O*-glucoside (5.6 μgL^{-1}) and cyanidin 3-*O*-glucoside (6.0 μgL^{-1}) from exogenously supplemented flavanones naringenin and eriodictyol, respectively. In this study, higher concentration of intermediates like dihydroflavonols and flavonols were found to accumulate in the fermentation broth (Yan et al., 2005). Similarly, two natural stilbene derivatives have been biosynthesized in practical amounts, resveratrol ($104.5 \pm 4.4 \text{ mgL}^{-1}$) and piceatannol ($13.3 \pm 0.3 \text{ mgL}^{-1}$), by supplementing natural phenylpropanoic acid precursors (*p*-coumaric acid and caffeic acid, respectively) from recombinant *E. coli* harboring plant stilbene biosynthesis genes *At4CL1* of *A. thaliana* and *AhSTS* of *A. hypogaea* (Watts et al., 2006). In the same way, 7-*O*-methyl aromadendrin was produced from exogenously added *p*-coumaric acid precursor in recombinant *E. coli* harboring flavonoid pathway genes *Pc4CL2*, *PhCHS*, *MsCHI*, *AtF3H* from different plant sources and a post modification gene 7-*O*-methyltransferase (*SaOMT*) from *S. avermitilis*. Introduction of ACC subunits, biotin ligase, and acetyl-CoA synthase genes from *Nocardia farcinica* resulted in the production of 2.7 mgL^{-1} of 7-*O*-methyl aromadendrin. When the precursor was naringenin, production was enhanced to 30 mgL^{-1} without any optimization of the malonyl-CoA availability (Malla et al., 2012).

Horinuchi and his group in 2007 designed *E. coli* based platforms for precursor-directed biosynthesis of various natural/unnatural flavonoids and stilbenoids. The flavonoid biosynthesis system was divided into three modules. First, the substrate synthesis step is catalyzed by *Le4CL-1* from *Lithospermum erythrorhizon*. Second, the polyketide synthesis step was catalyzed by either *GeCHS* from *G. echinata* for chalcone synthesis or *PlCHI* from *P. lobata* for flavanone synthesis, or *AhSTS* from *A. hypogaea* for stilbene synthesis. And third, the post-polyketide modification steps were catalyzed by *FSI* from *P. crispum* for flavones, and *F3H* and *FLS* from *Citrus* for flavonol synthesis. For increasing intracellular malonyl-CoA, two subunits of ACC (*accBC*) were also overexpressed from *C. glutamicum*. To these strains, 14 different chemically synthesized and naturally available carboxylic acid derivatives were fed to produce 87 different aromatic polyketides of which 36 were novel compounds including triketide and tetraketide pyrones (Fig. 5A). The scaled up reactions using cinnamic acid, *p*-coumaric acid, fluorocinnamic acid, furyl and thienyl cinnamic acid as starter precursors resulted in the production of 70-90 mgL⁻¹ of natural and 50-100 mgL⁻¹ of synthetic flavanones. While the production of natural and synthetic pyrones was limited to 3.6±1.1 mgL⁻¹ and 2.7±0.8 mgL⁻¹, respectively, the production of flavones was reported in the range of 25 mgL⁻¹ to 85 mgL⁻¹ while flavonols were produced in lower amounts. The production of stilbenes by supplementing above mentioned starter units was in the range of 50-170 mgL⁻¹ in comparison to other flavonoids (Katsuyama et al., 2007).

Koffas and colleagues also produced synthetic flavanone and flavone derivatives by supplementing synthetic phenylpropanoic acid derivatives derivatized by fluoro and amino functional groups at various positions of the phenolic ring. In addition to natural phenylpropanoic acids, *E. coli* harboring flavanone and flavone biosynthesis genes were fed

with (*E*) 2-fluoro-cinnamic acid, (*E*) 4-fluoro-cinnamic acid, (*E*) 2- cinnamic acid, (*E*) 3- cinnamic acid, and (*E*) 4-amino cinnamic acid. The flavanone producing strain generated pinocembrin, naringenin, eriodictyol, 5,7-dihydroxy-2'-fluoroflavanone, 5,7-dihydroxy-4'-fluoroflavanone, 5,7,2'-trihydroxy flavanone, 5,7,3'-trihydroxy flavanone, 4'-amino-5,7-dihydroxyflavanone whereas the flavone producing strain produced chrysin, apigenin, luteolin, 5,7-dihydroxy-2'-fluoroflavone, 5,7-dihydroxy-4'-fluoroflavone, 5,7,2'-trihydroxyflavone, and 4'-amino-5,7-dihydroxyflavone. The production titer of natural flavanones and flavones was considerably higher than synthetic derivatives (Leonard et al., 2008).

(Fig. 5 around here)

Similar to *E. coli*, biosynthesis of natural/synthetic flavonoids/isoflavonoids has been successfully achieved in *S. cerevisiae*. By using a combinatorial biosynthesis approach, Koffas and his colleagues were able to produce diverse kinds of synthetic flavonoids from *S. cerevisiae* by engineering plant flavonoid biosynthetic genes *4CL*, *CHS*, and *CHI* from various sources (Chemler et al., 2007). To the engineered strain, natural (*p*-coumaric acid, *m*-coumaric acid, *o*-coumaric acid) and chemically synthesized phenylpropanoic acids (*p*-fluorocinnamic acid, *o*-fluorocinnamic acid, *p*-aminocinnamic acid) were supplemented exogenously. As a result, (2*S*)-naringenin (60.04 mgL⁻¹), (2*S*)-3',5,7-trihydroxyflavanone (6.54 mgL⁻¹), (2*S*)-2',5,7-trihydroxyflavanone (6.36 mgL⁻¹), (2*S*)-4'-fluoro-5,7-dihydroxyflavanone (2.81 mgL⁻¹), (2*S*)-2'-fluoro-5,7-dihydroxyflavanone (6.54 mgL⁻¹), and (2*S*)-4'-amino-5,7-dihydroxyflavanone (15.82 mgL⁻¹) derivatives were produced. In addition, the recombinant flavanone biosynthetic pathway was also able to accept acrylic acid (*trans*, *trans*-muconic acid) to produce (2*E*)-[(2*S*)-5, 7-dihydroxy-4-chromanone] propenoic acid.

Additional expression of *F3H* in the same *S. cerevisiae* produced dihydroflavonol derivatives of aforementioned flavanones in a similar production range (0.75 to 63 mgL⁻¹) as flavanones (Chemler et al., 2007). Precursor directed biosynthesis of natural and unnatural isoflavonoids was also developed in *S. cerevisiae* by the same group using three enzymes *IFS*, *CPR*, and 2-hydroxyisoflavanone dehydratase (*HID*) from different plants. *IFS* encoding genes from five different plants were screened (*G. max*, *Trifolium pretense*, *G. echinata*, *Pisum sativum*, and *M. truncatula*) based on the production of genistein from naringenin. Finally, *GmIFS* and *GmHID* of *G. max*, and *CrCPR* of *C. roseus* harboring *S. cerevisiae* recombinant was used for the biotransformation of a variety of B ring modified flavanones which included 7 natural and 19 nonnatural hydroxyl, fluoro, chloro, bromo, methyl, methoxy, and ethoxy substituted flavanones (Fig. 5B). Among them, seven flavanones were not accepted as substrates for biotransformation to isoflavones. However, altogether 20 different isoflavones were produced using the engineered *S. cerevisiae* strain (Chemler et al., 2010b).

Besides *E. coli* and *S. cerevisiae*, one of the most commonly used hosts for production of various kinds of microbial polyketides is *S. venezuelae*. A strain deficient in pikromycin polyketide synthase was used as a heterologous host for production of flavonoids. A plasmid expressing *ScCCL* from *S. coelicolor* and *AtCHS* from *A. thaliana* was constructed and introduced into *S. venezuelae*. The resulting strain produced significantly small amounts of a racemic mixture of naringenin (0.2 mgL⁻¹) and pinocembrin (0.4 mgL⁻¹) from exogenously added *p*-coumaric acid and cinnamic acid, respectively. Further engineering of *ScCCL* and codon optimized *AtCHS* by cloning under separate *ermE** promoters enhanced the production of naringenin (4.0 mgL⁻¹) and pinocembrin (6.0 mgL⁻¹). Additional expression of codon optimized *MsCHI* from *M. sativa* under individual *ermE** promoter produced (2*S*)-flavanones;

however, the production was reduced by 10-fold. In another approach, codon optimized *AhSTS* from *A. hypogaea* was expressed together with *ScCCL* to produce stilbenes. The resulting strain produced 0.4 mgL⁻¹ of resveratrol and 0.6 mgL⁻¹ of pinosylvin from *p*-coumaric acid and cinnamic acid precursors, respectively (Park et al., 2009). The same group further engineered *S. venezuelae* by introducing codon-optimized *F3H* from *C. siensis* and *FLS* from *C. unshius*. The strain produced kaempferol and galanin from flavanone precursors naringenin and pinocembrin, respectively (Park et al., 2010). The integration of malonate assimilation pathway genes (*matB* and *matC*) in *S. venezuelae* genome and overexpression of flavanone (*ScCCL*, *AtCHS*, *AtCHI*) and flavone (*ScCCL*, *AtCHS*, *AtCHI*, and *PcFS1*) biosynthetic genes produced 35.6 mgL⁻¹ of naringenin and 44.1 mgL⁻¹ of eriodictyol as flavanones whereas 30.9 mgL⁻¹ of chrysin and 15.3 mgL⁻¹ of apigenin as flavones from *p*-coumaric acid and cinnamic acid (Park et al., 2011).

Finally, the biosynthesis of a novel, natural resveratrol dimer, resveratrol-*trans*-dihydrodimer, was recently reported through the use of a soybean peroxidase. The resveratrol-*trans*-dihydrodimer displayed antimicrobial activity against the Gram-positive bacteria *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus* (minimum inhibitory concentration (MIC) = 15.0, 125, and 62.0 µM, respectively) and against Gram-negative *Escherichia coli* (MIC = 123 µM, upon addition of the efflux pump inhibitor Phe-Arg-β-naphthylamide) (Mora-Pale et al., 2015).

2.6 System/Synthetic biology approaches

System biology encompasses the computational and mathematical modeling tools for biological systems that focus on complex interactions within a biological system (Herrgård et al., 2006; Stephanopoulos et al., 2004). For example, the study of interactions between the

components of a biological system such as enzymes and metabolites, or flux balance analysis for carbon and co-factors, to construct a model organism has become indispensable in metabolic engineering (Joyce and Palsson 2008; Oberhardt et al., 2009). Synthetic biology provides the tools that allow us to transplant the genes related to biosynthetic pathways from natural hosts into biotechnologically relevant hosts by precisely tuning and optimizing the gene expression in metabolic pathways. This is achieved by engineering molecular control elements in genetic device/vectors to accurately switch metabolic fluxes towards target pathway (Lee and Keasling, 2008; Young and Alper, 2010; Xu et al., 2012). In one approach, synthetic antisense RNAs (asRNAs) is used to downregulate the expression of pathway specific genes and optimize the flux of interest (Yang et al., 2015a). Design and construction of synthetic vectors and bio-parts for efficient cell factory development, innovative DNA assembly techniques for efficient assembly of combinatorial biosynthetic pathways simplify the major challenges of metabolic engineering for microbial production of natural products and commodity chemicals (Gibson et al., 2009; Jeong et al., 2012; Markham and Alper 2015; Xu et al. 2012). Both systems and synthetic biology accelerate the understanding of biological systems to engineer cells for novel functions. Under this subsection, we will focus on applications of system/synthetic biology tools for improved production of flavonoids and derivatives.

2.6.1 Computational designing/modelling for cofactor/precursor enhancement

Because of cellular complexity and interconnectivity, the identification of genetic targets for modification for the purpose of redirecting metabolic fluxes is difficult to perform by simple metabolic pathway inspection. However, rapid advances in systems and synthetic biology aid in addressing this engineering conundrum (Stephanopoulos et al., 2004; Lanza et al., 2012). A

Cipher of Evolutionary Design (CiED) was developed based on the genetic algorithm and was used for strain design using a previously published *E. coli* metabolic model (*EciJR904* GSM/GPR) (Reed et al., 2003). This strain design method allowed the development of a new *E. coli* genotype with enhanced carbon flux toward malonyl-CoA and other cofactors. Based on the CiED algorithm, deletion of citrate cycle genes *sdhCDAB* encoding for succinate dehydrogenase and citrate lyase (*citE*), the amino acid transporter *brnQ*, and the pyruvate consuming enzyme acetaldehyde dehydrogenase (*adhE*), combined with overexpressions in the CoA and malonyl-CoA biosynthetic pathways, generated an efficient genotype for the production of flavanones. Among 11 engineered strains, *E. coli* strain (Z40NAC) deficient in *sdhA*, *adhE*, *brnQ* and *citE* harboring flavanone biosynthesis pathway genes (*Pc4CL2*, *PhCHS*, and *MsCHI*) and overexpression of cofactor and assimilation pathways (ACC complex and biotin ligase (*BirA*) from *P. luminescens*, *coaA* encoding for pantothenate kinase and *acs* from *E. coli*) produced 270 mgL⁻¹ of naringenin and 150 mgL⁻¹ of eriodictyol when feeding with *p*-coumaric acid and caffeic acid, respectively (Fowler et al., 2009). Following production of flavanone by enhancing the malonyl-CoA pool in cell cytosol by using the CiED based *E. coli* model, the same tool was used to develop *E. coli* genotypes generating improved reduced nicotinamide adenine dinucleotide phosphate (NADPH). This strain was applied to leucoanthocyanidin and (+)-catechin biosynthesis in which NADPH serves as essential cofactor. Twenty-two *E. coli* strains deficient in a single and a combination of various genes were generated, and *E. coli* BL21 deficient in glucose-6-phosphate isomerase (*pgi*), phospholipase A or lysophospholipase L (*pldA/B*) and phosphoenolpyruvate carboxylase (*ppc*) resulted in production of 817 mgL⁻¹ leucocyanidin while using *DuLAR* from *D. uncinatum* and supplementing 5 mM of dihydroquercetin. The same *E. coli* mutant

(*E. coli* BL/ Δ *pgi* Δ *pldA* Δ *ppc*) overexpressing *DuLAR* and *AaDFR* from *A. andraeanum* produced 38.8 mgL⁻¹ of (+)-catechin upon supplementation of 1 mM of dihydroquercetin. The final titer of leucocyanidin and (+)-catechin was 4-fold improvement in comparison to wild type *E. coli* overexpressing respective genes. The intracellular measurement of [NADPH] level and [NADPH]/[NADP⁺] ratio was found to be significantly improved in the *E. coli* BL/ Δ *pgi* Δ *pldA* Δ *ppc* strain and determined to be one of the superior strains for biosynthesis of compounds utilizing NADPH (Chemler et al., 2010a). Similarly, the OptForce integrated computational approach was applied to design an *E. coli* BL21StarTM strain with improved flux towards malonyl-CoA. The great benefit of OptForce is that, in addition to deletions it can also identify targets for deletions and for downregulations. The knockout of fumerase encoding gene (*fumC*) and succinyl-CoA synthetase encoding gene (*sucC*) and overexpression of *accABCD*, phosphoglycerate kinase encoded by *pgk*, and pyruvate dehydrogenase complex encoded by *aceEF* and *lpdA* genes produced four-fold higher intracellular malonyl-CoA than wild type strain. The same strain upon expression of additional genes for naringenin biosynthesis (*Pc4CL2*, *PhCHS* and *MsCHI*) produced 474.2 mgL⁻¹ of naringenin from exogenously supplemented *p*-coumaric acid (Xu et al., 2011). The same model was also used for the production of 1.6 g/l of resveratrol by overexpressing *A. thaliana* *At4CL-2* and *V. vinifera* *VvSTS* genes downstream of the gap promoter (P_{GAP}) (Bhan et al., 2013). These are the successful examples of applications of systems biology tools for flavonoid production. It is important to note that in addition to overexpressions and deletions, OptForce predicted a number of downregulations for improving flavanone production, such as the downregulation of *fumB*, *fumC* and *acnA*. Some of these downregulations, together with the downregulation of FadR (a transcription factor regulating fatty acid biosynthesis)

were recently tested individually using CRISPRi/dCas9 and demonstrated the ability to increase flavanone production (Cress et al., 2015a).

2.6.2 Modular metabolic engineering

Metabolic engineering involves a series of genetic manipulations to increase the robustness of so-called “bio-factories”. For the production of complex pharmaceuticals or nutraceuticals, long metabolic pathways are often involved. There are several cloning techniques to incorporate pathway specific genes to build a complete recombinant vector or integration of multiple genes into host chromosomes for production. However, these strategies are not sufficient in industrial biotechnology for targeted chemical/compounds production. Recently, modular metabolic engineering approach has been employed for production of various compounds like taxol (Ajikumar et al., 2010), fatty acids (Xu et al., 2013), protein (Gu et al., 2015), fuels and chemicals (Tseng and Prather, 2012) and many other compounds and commodities including flavonoids (Jones et al., 2015a; Jones et al. 2015b; Cress et al., 2015b). Wu and colleagues have made progress on flavonoid production enhancement by modular metabolic engineering of the flavonoid biosynthetic pathway. For example, the entire (2S) pinocembrin biosynthesis pathway genes from glucose were divided into four different modules. The first module produces phenylalanine from D-glucose and contains overexpression of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHPS) *aroF* (*E. coli* K12) and feedback inhibition resistant chorismate mutase/prephenate dehydratase (*pheA^{fbr}*). The second module produces cinnamoyl-CoA from L-phenylalanine and contains *RgPAL* (*R. glutinis*) and *Pc4CL*. The third module produces (2S) pinocembrin *PhCHS* and *MsCHI*. The final module produces a pool of malonyl-CoA from malonate (*matB* and *matC* from *R. trifolii*). All genes were assembled under the T7 promoter in four different vectors

with different copy numbers. Different combinations of the modules were tested for the optimum production of (2*S*) pinocembrin by constructing four strains. The optimally balanced pathway produced 40.02 mgL⁻¹ of final product from glucose without supplementation of precursors in the medium (Wu et al., 2013a). Similarly, the coumaroyl-CoA producing module, malonyl-CoA generating module and (2*S*)-naringenin producing module were constructed by cloning the same set of (2*S*)-pinocembrin producing genes. *RgTAL* from *R. glutinis* exhibited the highest *in vitro* enzymatic activity towards L-tyrosine, and was selected instead of *RgPAL*. *Pc4CL*, *PhCHS*, *MsCHI*, *matB* and *matC* were assembled to construct synthetic flavonoid biosynthesis pathway in *E. coli* to produce (2*S*)-naringenin. Each module was assembled in plasmids with different copy numbers and promoter strengths (T7 or Ptrc). Different combinations of modules were assessed to identify an optimally balanced pathway. The optimal strain produced 90.59 mgL⁻¹ of naringenin from L-tyrosine. Additional modules overexpressing feedback resistant genes DAHP synthase *aroG*^{fbr} and *tyrA*^{fbr} produced 100.64 mgL⁻¹ of naringenin from D-glucose, the highest production titer of naringenin in *E. coli* from D-glucose (Wu et al., 2014b). Multivariate modular metabolic pathways were also constructed for resveratrol production from simple precursor like L-tyrosine. Efficient bioconversion of L-tyrosine to resveratrol is one of the rate limiting steps (Lim et al., 2011) a problem that has been partially addressed by engineering the resveratrol biosynthesis pathway using the multivariate modular engineering approach. More specifically, the resveratrol biosynthetic pathway was divided into three modules: the first module was the coumaroyl-CoA producing module containing *RgTAL* and *Pc4CL*, the second module was the resveratrol synthesis module containing *VvSTS* from *V. vinifera* and the third module was the malonyl-CoA producing module containing *matB* and *matC*. Each module was constructed

under two different promoters having different strengths (T7 and Ptrc) in four different copy number plasmid backbones (pETDuet-1, pRSFDuet-1, pACYCDuet-1, and pCDFDuet-1). Eighteen different combinations of the three modules were checked for production of resveratrol from L-tyrosine. The best combination of the three modules (first module genes under Ptrc promoter in CDF origin of replication plasmid, second module under T7 promoter in pBR322 origin of replication plasmid backbone and third module under T7 promoter in p15A origin of replication containing plasmid) produced maximum of 35.02 mgL⁻¹ of resveratrol (Wu et al., 2013b).

2.6.3 Vector design

A series of genetic manipulation is required to balance metabolic flux in bacteria during flavonoid biosynthesis. Synthetic biology makes the control and manipulation of biological systems easier by constructing complex biological devices/vectors that allow the easy cloning of multiple genes involved in a biosynthetic pathway (Xu and Koffas, 2013). Vectors are common tools to carry genetic materials into the host. Several essential features of a vector can be engineered, such as compatible origin of replication so that multiple vectors can be co-transformed in the same host strain, compatible vector/gene copy number of plasmid, promoter strength, and ribosome binding sites to ensure higher product titer (Ajikumar et al., 2010; Wu et al., 2013b).

Synthetic vectors have been designed so that they allow the construction of artificial biosynthetic pathways for fine tuning and higher titer production of various flavonoids and their derivatives. The ePathBrick vector system has been developed for fine-tuning of gene expression and modular assembly of pathway genes and transcriptional components and has been applied for the enhanced production of the flavanone naringenin (Xu et al., 2012). In

this report, the pseudo-operon gene structure (each pathway gene was under the control of an independent promoter while all mRNA transcripts shared the same terminator) consisting of the multigene pathway for naringenin biosynthesis (*Pc4CL-PhCHS-MsCHI*) and malonyl-CoA biosynthesis (*accD-accC-accB-accA*) led to the highest naringenin production from all gene configurations tested (36.7 mgL⁻¹ without ACC complex encoding genes and ~80 mgL⁻¹ with ACC genes). Similarly, employing the same ePathBrick vector system, (+)-catechin was produced at a titer of 910.9 mgL⁻¹ from 1.0 gL⁻¹ of eriodictyol in batch culture (Zhao et al., 2015). In another independent study, seven NDP-sugar biosynthesis pathway genes were cloned in monocitronic form under the independent T7 promoter and mRNA terminator for efficient production of flavonol glycosides. Validation of these synthetic sugar cassettes was accomplished by biotransformation of flavonols and was found to be efficient enough to achieve the production of 392.6 mgL⁻¹ of fisetin 3-*O*-glucoside and 342 mgL⁻¹ of fisetin 3-*O*-rhamnoside. These cassettes were able to produce similar yield with other flavonols (quercetin, kaempferol, myricetin) (Chaudhary et al., 2013; Parajuli et al., 2015).

Recently, bio-sensor plasmids have been designed to efficiently detect the flavonoid metabolites at the single cell level by engineering transcriptional regulators from *Herbaspirillum seropedicae* and *Bacillus subtilis* (Siedler et al., 2014). Knocking down of pathways that diverge carbon from the flavonoid pathway such as fatty acid biosynthetic genes (*fabD*) was achieved by employing synthetic antisense RNA technology and resulted in high intracellular concentration of malonyl-CoA in *E. coli*. The recombinant *E. coli* harboring plant derived naringenin and resveratrol biosynthesis genes produced 1.70 fold higher resveratrol (268.2 mgL⁻¹) and 1.53 fold higher naringenin (91.31 mgL⁻¹) compared to the control strain lacking antisense RNA coding plasmids (Yang et al., 2015a).

2.6.4 Promoter engineering

Tuning of multiple gene expression at the macroscopic level in practical applications can be achieved through promoter engineering (Alper et al., 2005). Promoters have been used to modify gene expression for decades, especially for tuning the expression of heterologous proteins. These promoters possibly minimize the metabolic burden and allow regulation of expression at varying inducer levels in cultured cells (Lee and Keasling, 2008). Auto inductive and inducible (beside IPTG) promoters have found extensive applications in recent years; the arabinose inducible promoter, the propionate inducible promoter, constitutive promoters, promoters induced by starvation of an essential nutrient, promoters induced upon entry into stationary phase, the effector (flavonoid) inducible promoters are all promoters to be considered for gene expression that would result in high yield production (Khlebnikov et al., 2001; Keasling, 2012; Lee and Keasling, 2008; Siedler et al., 2014). Application of promoter engineering in flavonoid biosynthesis pathway in *E. coli* hosts made possible the synthesis of 100.64 mgL⁻¹ of naringenin from glucose (Wu et al., 2014b), 2.3 g/l of resveratrol from *p*-coumaric acid (Lim et al., 2011), 40.02 mgL⁻¹ of (2*S*)-pinocembrin from glucose (Wu et al., 2013) and 107 mgL⁻¹ of eriodictyol from L-tyrosine (Zhu et al., 2014). A hybrid *cis*- and *trans*- regulatory promoter was designed from *B. subtilis*, which responds to a broad concentration range of malonyl-CoA (rate limiting precursor of value-added pharmaceuticals and small molecules like flavonoids) during metabolic process, resulting in the dynamic control of malonyl-CoA associated fluxes (Xu et al., 2014).

2.6.5 Ribosome Binding Sites

Unlike promoters, the ribosome binding sites (RBSs) are also an essential regulatory element

which directly affects translation efficiency of the gene (Salis et al., 2009). The fundamental challenge in all kinds of metabolic engineering projects is to maximize the product yield based on the “central dogma” (transcription, translation and RNA processing) (Boyle and Silver, 2011). Optimization of RBSs using computational approaches mediates tunable gene expression providing rational control over protein production so that bottlenecks can be identified and removed at the translational level (Salis et al., 2009; Wu et al. 2014a; Xu et al., 2012). The RBS calculator allows the construction of customizable RBSs for a given gene sequence (Boyle and Silver, 2011; Salis et al., 2009).

The importance of RBS engineering was shown in the expression of an artificial gene cluster containing *RrPAL*, *ScCCL*, and *GeCHS* that resulted in efficient gene expression and synthesis of naringenin chalcone (Hwang et al., 2003). Enhancement in production of (-)-epicatechin (0.01 mgL^{-1}), (-)-epicatechin gallate (0.36 mgL^{-1}), (+)-catechin hydrate (0.13 mgL^{-1}) and (-)-catechin gallate (0.04 mgL^{-1}) was achieved by using different RBSs in different constructs (Umar et al., 2012). In a later study, after the application of synthetic sRNA modulation on metabolic engineering, combinatorial RBS optimization and promoter strength resulted in the development of an efficient *E. coli* strain that produced 2 gL^{-1} of L-tyrosine, an alternative precursor for flavonoid biosynthesis (Na et al., 2013).

3. Post-modification of flavonoids

Post-modifications of natural products are usually carried out by a simple biotransformation approach using *Streptomyces* strains, fungal mycelia, and plant cells (Shimoda et al., 2010; Kim, 2010; da Silva and Rodrigues-Fo, 2010; Ascari et al., 2011; Wu et al., 2015). Actinomycetes and eukaryotic cells have a wide number of natural product-modifying native enzymes. The supplementation of purified natural products, such as flavonoids, in these

growing cells leads to spontaneous modification of the compounds by the cells' own available cofactor(s) and enzymes (Cao et al., 2014). Since this strategy of modification of natural compounds is simple and inexpensive, the approach can be scaled up to the industrial level upon optimization of the biotransformation conditions for high level production of target compounds. Importantly, the production of the desired products should be significantly high in order for the resulting process to be commercially viable. However, the drawback of this method lies with the production of a wide range of unexpected byproducts and as a result, the difficulty of purification. To minimize the side products and accomplish regioselective modification of flavonoids and other natural products, model organisms such as *E. coli*, *Streptomyces* or *S. cerevisiae* are engineered by introducing a partial or entire biosynthetic pathway gene clusters of intracellular co-factors/precursors along with post modification genes (Luo et al., 2015; Sun et al., 2015; Zhang et al., 2015; Kim et al., 2015a). Exogenously supplemented compounds are biotransformed by various enzymes upon intake by the cell. Most of the post modifications of flavonoids are carried out by enzymes such as glycosyltransferases (GTs), *O*-methyltransferases (OMTs), cytochrome P450s (CYP), and prenyltransferases (PTs) which will be discussed herein. The selected enzymes involved in post-modification of flavonoids are highlighted in Table 4 with their sources and substrate promiscuity and nature of modification reactions. Besides these four major post-modification reactions, flavonoids are found to be modified by other diverse reactions in whole cell biotransformation while using microbial cells mainly fungi or actinomycetes. Those post-modification reactions are dehydroxylation (Lin et al., 2014), deglycosylation (Dajanta et al., 2009; You et al., 2010; Liu et al., 2013, Lin et al., 2014), hydrogenation (Correa et al., 2011; Stompor et al., 2013), halogenation (Xiang et al., 2010), and sulfation (Ibrahim et al., 2010;

Araujo et al., 2013). These post-modifications of flavonoids are still understudied and require extensive exploitation. Since this review is not focusing on simple biotransformation of flavonoids using plants, actinomycetes and animal cells, these modifications are not emphasized herein. Readers interested in modifications of flavonoids by simple biotransformation approach are requested to go through recent review by Cao et al. (2014).

3.1. Glycosylation

Glycosylation, one of the most abundant post modification processes, is the process by which a sugar unit is transferred from an activated sugar nucleotide diphosphate sugar to an acceptor molecule. Moreover, glycosylation determines the chemical complexity and diversity of natural products, and brings physical as well as biological changes in the molecule (Weymouth-Wilson, 1997). The plant and bacterial GTs that belong to GT1 family of proteins usually catalyze a direct displacement following the SN₂-like mechanism (Lairson et al., 2008; Wang, 2009). These GTs have been used widely for the post-modification of flavonoids by engineered microbial cells. Most of the flavonoid aglycones are biotransformed to respective glycosides by *E. coli* having engineered nucleotide sugar pathways. The sugar pathways that have been engineered in *E. coli* for the production of flavonoid glycosides are presented in Fig. 6 along with the structures of the biosynthesized flavonoid glycosides.

Several nucleotide sugar biosynthetic pathways are engineered in *E. coli* for regioselective biotransformation and high titer production of flavonoid glycosides. For example, UDP-glucose dehydrogenase (*CalS8*) and UDP-glucuronic acid decarboxylase (*CalS9*) from *Micromonospora echinospora* spp. *calichensis* were expressed together with an integrated copy of *E. coli* glucose-1-phosphate uridylyltransferase (*galU*) in the background strain *E. coli* BL21(DE3) deficient of glucose phosphate isomerase (*pgi*) for excess intracellular

availability of UDP-xylose along with 7-*O*-glycosyltransferase (*ArGt-4*) from *A. thaliana*. The engineered strain was fed with naringenin to produce naringenin 7-*O*-xyloside (Simkhada et al., 2009). More recently, quercetin 3-*O*-xyloside (42.5 mgL⁻¹) has been solely produced by biotransformation of quercetin in *E. coli* with an engineered UDP- α -D-glucose pathway in which glucose-6-phosphate utilizing pathway genes such as *pgi*, glucose-6-phosphate 1-dehydrogenase (*zwf*), and UDP- α -D-glucose hydrolase (*ushA*) were blocked and the entire UDP-xylose biosynthetic pathway genes from glucose-1-phosphate were overexpressed (Pandey et al., 2013b). Han et al. (2014) also synthesized the same product to a higher scale of 150 mgL⁻¹ by overexpressing UDP-xylose synthase (*uxs*), UDP-glucose 6-dehydrogenase (*ugd*), and *A. thaliana* *AtUGT78D3* in a UDP-4-amino-4-deoxy-L-arabinose (L-Ara4N) formyltransferase / UDP-glucuronic acid C-4"-decarboxylase (*arnA*) deleted *E. coli* mutant. Similarly, the pools of TDP-L-rhamnose and TDP-D-allose sugars were developed separately by expressing the respective sugar biosynthetic pathway genes from different bacterial sources in the *pgi*-deleted *E. coli* BL21 (DE3). The recombinant strains overexpressing additional *A. thaliana* GT (*ArGt-3*) were able to biotransform both quercetin and kaempferol to 3-*O*-rhamnosyl quercetin (24 mgL⁻¹) and 3-*O*-rhamnosyl kaempferol (12.9 mgL⁻¹), while 3-*O*-allosyl quercetin was produced in trace amount (Simkhada et al., 2010). In another similar recent report, *E. coli* BL21 (DE3) carrying three genes, *ugd*, *arnA*, and *arnB* (UDP-L-Ara4-*O*-C-4" transaminase) along with glycosyltransferase *AtUGT78D3* from *A. thaliana*, produced quercetin-3-*O*-4-deoxy-4-formamido-L-arabinose (70 mgL⁻¹) from quercetin (Kim et al., 2010a). Other examples of biotransformation of aglycones to glycosides using a similar nucleotide sugar pathway engineering approach in *E. coli* include production of quercetin 3-*O*-*N*-acetylglucosamine (380 mgL⁻¹) (Kim et al., 2012b), quercetin

3-*O*-arabinoside (160 mgL⁻¹) (Han et al., 2014), quercetin 3-*O*-6-deoxytaloside (98 mgL⁻¹) (Yoon et al., 2012), quercetin 3-*O*-galactoside (280 mgL⁻¹), quercetin 3-*O*-glucuronide (687 mgL⁻¹), and luteolin 7-*O*-glucuronide (300 mgL⁻¹) (Kim et al., 2015b) (Fig. 6). Roepke and Bozzo also overexpressed the UDP-L-rhamnose biosynthetic pathway and biotransformed exogenously fed quercetin 3-*O*-glucoside to quercetin 3-*O*-glucoside-7-*O*-rhamnoside (20 mgL⁻¹) by using *A. thaliana* flavonol 7-*O*-rhamnosyltransferase (*UGT89C1*) (Roepke and Bozzo, 2013). Two quercetin bisglycosides, quercetin 3-*O*-glucoside-7-*O*-rhamnoside (67 mgL⁻¹) and quercetin 3,7-*O*-bisrhamnoside (67.4 mgL⁻¹) were also produced using two different combinations of three GTs (Kim et al., 2013b). Recently, we have also produced kaempferol 3-*O*-glucoside (109.3 mgL⁻¹) from naringenin by introducing naringenin modifying genes (*AtF3H* and *AtFLS1* from *A. thaliana*) and UDP- α -D-glucose pathway genes overexpressing background strain of *E. coli* BL21(DE3)/ $\Delta pgi\Delta zwf\Delta ushA$ (Malla et al., 2013). Moreover, a newly constructed *E. coli* BL21(DE3)/ $\Delta pgi\Delta zwf\Delta galU$ mutant was engineered to generate three different dTDP-amino-deoxy sugars (dTDP-4,6 dideoxy 4-amino-D-galactose, dTDP-4,6 dideoxy 4-amino-D-glucose, and dTDP-3,6 dideoxy 3-amino-D-galactose) by overexpressing dTDP 4-keto 4,6-dideoxy glucose intermediate modifying sugar aminotransferases from various sources. Over expression of an *A. thaliana* GT transferred 4,6 dideoxy 4-amino-D-galactose and 3,6 dideoxy 3-amino-D-galactose sugar moieties to 3-OH of exogenously supplemented quercetin and kaempferol. Altogether, four novel flavonols with four novel conjugated aminodeoxy sugars were successfully generated by this biotransformation approach (Pandey et al., 2015). In our recent report (Parajuli et al., 2015), we produced glucoside and rhamnoside derivatives of fisetin by engineering respective sugar pathway genes in a mono-cistronic fashion. This approach of

biotransformation resulted in significantly higher titers of target sugar conjugated fisetin glycosides in which 392.6 mgL⁻¹ of fisetin 3-*O*-glucoside and 342 mgL⁻¹ of fisetin 3-*O*-rhamnoside were produced in a lab scale fermentor. Likewise, myricetin 3-*O*-rhamnoside (25.8 mgL⁻¹), apigenin-7-*O*-glucoside (39.28 mgL⁻¹), baicalein-7-*O*-glucoside (33.19 mgL⁻¹) have also been produced using *Arabidopsis* GTs in UDP- α -D-glucose-producing engineered *E. coli* cells (Thuan et al., 2013a; 2013b). Using the same strain that over-produces UDP- α -D-glucose, and also expressing isoflavonoid specific GT (*GmIF7GT*), and 6''-*O*-malonyltransferase (*GmIF7MaT*) from *G. max*, genistein 7-*O*-glucoside (76.82 mgL⁻¹), genistein 7-*O*-glucoside-6''-*O*-malonate (7.37 mgL⁻¹), daidzein 7-*O*-glucoside (67.65 mgL⁻¹), and daidzein 7-*O*-glucoside-6''-*O*-malonate (6.23 mgL⁻¹) were produced by feeding genistein and daidzein aglycones (Koirala et al., 2014a). Similarly, the use of a GT from *Bacillus licheniformis* (YjiC), Pandey et al. (2014a) resulted in the production of several glucosides of genistein, daidzein, formononetin, and biochanin A (Fig. 6).

(Fig. 6 and Table 4 around here)

Glucoside derivatives of flavonoids have also been produced by using *E. coli* cells harboring only GT without engineering UDP-glucose biosynthesis pathway genes. For example, diverse quercetin glucosides (quercetin 3-*O*-glucoside, quercetin 7-*O*-glucoside, quercetin 3'-*O*-glucoside, and quercetin 4'-*O*-glucoside) have been produced by simple biotransformation of quercetin using *E. coli* cells harboring different *Arabidopsis* GTs (Lim et al., 2004). Similar approaches were also used by other groups to produce kaempferol 3-*O*-glucoside (44.8 mgL⁻¹) and quercetin 3-*O*-glucoside (46.4 mgL⁻¹) (Kim et al., 2006c), luteolin-4'-*O*-glucoside (10.8 mgL⁻¹), luteolin 7-*O*-glucoside (6.52 mgL⁻¹), genistein 7-*O*-glucoside (20 mgL⁻¹), biochanin A 7-*O*-glucoside (13 mgL⁻¹) (He et al., 2008), and isorhamnetin 3-*O*-glucoside (39.7 mgL⁻¹)

(Kim et al., 2010b) from respective aglycones. Four different phloretin glucosides (phloretin 4'-*O*-glucoside (60.1 mgL⁻¹), phloretin 2'-*O*-glucoside (9.59 mgL⁻¹), phloretin 4,4'-*O*-diglucoside (35.96 mgL⁻¹), phloretin 2',4',4'-*O*-triglucoside (162.8 mgL⁻¹)) were also produced using an *E. coli*/Δ*pgi* Δ*zwf* Δ*ushA* harboring YjiC GT from *B. licheniformis* (Pandey et al., 2013a) (Fig. 7).

(Fig. 7 around here)

3.2 Methylation

Methylation is another common post modification of many secondary metabolites. Plant originated secondary metabolites, especially flavonoids, are methylated by S-adenosyl-L-methionine (SAM)-dependent caffeoyl-CoA *O*-methyltransferases (OMTs). However, a few of the microbial originated SAM-dependent OMTs are also found to methylate plant flavonoids. In this section, microbial biotransformation of flavonoids using engineered *E. coli*, yeast, and *Streptomyces* to methylated derivatives will be discussed.

A *G. max* originated *O*-methyltransferase (*SOMT-2*) was functionally overexpressed in *E. coli* and used for the biotransformation of various exogenously added flavonoids (apigenin, naringenin, and quercetin) and isoflavonoids (daidzein, genistein) which were stoichiometrically converted to 4'-*O*-methylated derivatives (Kim et al., 2005a). By using poplar OMT (*POMT-7*), Ahn and his group biotransformed a range of flavonoids, including flavonols (quercetin, kaempferol, isorhamnetin), flavones (apigenin, luteolin), and flavanone (eriodictyol, naringenin) to produce respective 7-*O*-methylated derivatives (Kim et al., 2006a). The same group also studied co-expression of two regiospecific OMTs, rice originated *ROMT-9* and *SOMT-2* in *E. coli* and produced regiospecifically methylated 3'-

methyated and the 3',4'-dimethyated quercetin derivatives (Kim et al., 2005b). A similar approach of coexpression of two previously characterized regioselective OMTs, *POMT-7* regiospecific for 7-OH and *ROMT-9* regiospecific for 3'-OH was used for biotransformation of flavonoids in *E. coli*. The recombinant strain was fed with flavonoids (quercetin, and luteolin) for production of specific compounds; however a mixture of multiple methyated compounds was produced. For example, 3' and 4' methyated derivatives of luteolin (3'-*O*-methyl luteolin, 3',4'-*O*-dimethyl luteolin, 7,3'-*O*-dimethyl luteolin, and 7,3',4'-*O*-trimethyl luteolin) and quercetin (3'-*O*-methyl quercetin, 3',4'-*O*-dimethyl quercetin, 7,3'-*O*-dimethyl quercetin, and 7, 3',4'-*O*-trimethyl quercetin) (Fig. 8) were all produced in the biotransformation reaction mixture altering the regioselectivity of *POMT-7* from 7- to the 4'-OH groups (Kim et al., 2008).

Beside plant OMTs, *Streptomyces* derived OMTs were also used for the biotransformation of flavonoids. An OMT from *S. avermitilis* MA-4680 (*SaOMT-2*) was expressed in *E. coli*. The recombinant strain was able to transfer a methyl group regiospecifically at the 7-OH group of isoflavones (daidzein, genistein), flavonols (kaempferol, quercetin, isorhamnetin), flavones (apigenin), and flavanones (naringenin) (Kim et al., 2006b). Another OMT from *S. peucetius* ATCC 27952 (*SpOMT2884*) was also functionally overexpressed in *E. coli* and used for the biotransformation of various flavonoids such as flavones (7,8-dihydroxyflavone, luteolin), flavonols (quercetin, rutin), flavanones (naringenin), and isoflavonoids (daidzein, formononetin). One preferred substrate, 7,8-dihydroxyflavone was converted to 7-hydroxy-8-methoxyflavone at relatively higher rate. Optimization of substrate concentration, time, media parameters and scale-up using a 3-L fermentor produced 52.57 mgL⁻¹ of 7-hydroxy-8-methoxyflavone from 200 μ M of 7, 8-dihydroxyflavone within 12h (Koirala et al., 2014b).

(Fig. 8 around here)

3.3 Hydroxylation

Hydroxylation of flavonoids is also an important post modification for the biosynthesis of a diverse array of flavonoids. In order to generate diversity in structural chemistry of flavonoids and to produce novel therapeutically or nutraceutically important compounds, flavonoids are hydroxylated by various microbial biotransformation approaches. However, very limited studies have been carried out to produce hydroxylated derivatives of flavonoids using microbial systems. Whole cell biotransformation of daidzein, one of the phytoestrogen isoflavones, using *Nocardia farcinica* IFM 10152 resulted in production of 3',4',7-trihydroxyisoflavone, 4',6,7-trihydroxyisoflavone, and 4',7,8-trihydroxyisoflavone. Moreover, the study identified two key cytochrome P450 (CYP450) monooxygenases (*nfa12130* and *nfa33880*) with their native ferredoxins and ferredoxin reductase partner responsible for the hydroxylation among 27 different CYP450s. The recombinant *E. coli* expressing the same set of CYPs was also able to produce some of the hydroxylated derivatives of daidzein (Choi et al., 2009). A similar approach of whole cell biotransformation of two isoflavones (daidzein and genistein) was carried out using *S. avermitilis* MS-4680. The strain biotransformed daidzein to 3',4',7-trihydroxyisoflavone and genistein to 3',4',5,7-tetrahydroxyisoflavone by regiospecifically hydroxylating at the ortho-position with an average of 16% molar conversion yield after 24 h of reaction (Roh et al., 2009a).

A recombinant *E. coli* strain, constructed by overexpressing the *B. subtilis* 168 *CYP107H1* along with putidaredoxin reductase (*camA*) and putidaredoxin (*camB*) from *Pseudomonas putida* as the redox partners, hydroxylated exogenously fed daidzein regiospecifically at 3'-ortho-position to yield 7,3',4'-trihydroxyisoflavone (Fig. 8). However, the conversion yield

was relatively small (0.6%) (Roh et al., 2009b). The same group also used *S. avermitilis* MA4680 as an expression host for the expression of *CYP107Y1* (SAV2377), *CYP125A2* (SAV5841) and *CYP107P2* (SAV4539) originally derived from the same strain. The recombinant *S. avermitilis* strains overexpressing *CYP107Y1*, *CYP125A2*, and *CYP107P2* biotransformed genistein, chrysin and apigenin to 3',4',5,7,-tetrahydroxyisoflavone, B-ring hydroxylated 5,7-dihydroxyflavone and 3',4',5,7,-tetrahydroxyflavone, respectively. The molar conversion was 25% for genistein, 12% for chrysin, and 10% for apigenin. *E. coli* expressing the same enzymes with *P. putida* redox partners (*PdR/Pdx*) also transformed the same set of flavonoids with 3-4 fold lower conversion rate (Pandey et al., 2011).

3.4 Prenylation

Prenylated flavonoids, conjugated with lipophilic prenyl side-chain (5-carbon (dimethylallyl), 10-carbon (geranyl) or 15-carbon (farnesyl) have low abundance in nature. They have many applications as nutraceuticals and pharmaceuticals, however their chemical synthesis is tedious with limited production yield (Botta et al., 2005a). Thus, microbial biotransformation is used for the production of prenylated derivatives using prenyltransferases (PTs) from various sources (Botta et al., 2005b). A thorough recent review covering the topic of prenylated flavonoids and their importance and biological activities has recently been published by Yang et al. (2015b). Most of the prenylated flavonoids are produced by biotransformation using plant and fungal whole cells. Use of engineered microbial cells for production of prenylated flavonoids has not yet been explored.

A PT (*Orf2*) from *Streptomyces* sp. strain CL190 was identified to transfer the geranyl group to various flavonoid compounds such as daidzein, formononetin, genistein, fisetin, naringenin, and resveratrol (Kuzuyama et al., 2005). The same *Orf2* (renamed *NphB*) and *SCO7190*, a

NphB homolog from *S. coelicolor* A3(2) was used for the chemoenzymatic synthesis of various prenylated flavonoids (Kumano et al., 2008). Similarly, another PT from *S. niveus*, *NovQ* was also characterized to transfer dimethylallyl group to phenylpropanoids and B-ring of flavonoids (Ozaki et al., 2009). However, none of these enzymes was used for *in vivo* production of prenylated flavonoids. A naringenin 8-dimethylallyltransferase (*SfN8DT-1*) from *Sophora flavescens* which transfers a prenyl group at the 8-position of naringenin was expressed in yeast strain W303-1A that was deficient in coenzyme Q2 4-hydroxybenzoate polyprenyltransferase (*COQ2*) (Fig. 8). The transgenic yeast strain produced a maximum of 566 μgL^{-1} of 8-dimethylallyl naringenin from exogenously added 0.2 mM naringenin (Sasaki et al., 2009).

4. Conclusions

Flavonoids are ubiquitous in the plant kingdom, have diverse chemical structures, and their applications in human health have been widely studied. The biosynthetic pathways of flavonoids have also been intensively studied and characterized to define the role and mechanism of each gene involved. Moreover, a large number of flavonoids have been identified and continue to be isolated from plants (Veitch and Grayer, 2011), some of which are recognized as potential lead compounds for onset of diseases (<https://clinicaltrials.gov/>; Xiao et al., 2014). In addition, the flavonoids and their derivatives have also been broadly used in functional foods, nutraceuticals, and cosmetics. The current market of flavonoids is dependent upon availability of their plant sources, which is affected by seasonal variations and involves costly, inefficient, and environmentally unfriendly extraction processes. Thus, implanting heterologous plant flavonoid biosynthesis pathways in microbial hosts is an alternative method of production. Application of recent molecular biology and system/synthetic biology tools has created the potential for the biosynthesis of a multitude of flavonoids and unnatural derivatives from genetically tractable microbial hosts such as *E. coli*, *Streptomyces* and *S. cerevisiae*. However, low microbial production titer yields of flavonoids from cheap carbon sources is one of the limiting factors for industrial scale-up and commercial production of flavonoids using recombinant microbial cells. The identification of bottlenecks, fine tuning/optimization of flavonoid biosynthetic pathways, and simultaneous balance of metabolic fluxes by rewiring native precursors/cofactors biosynthesis pathways of host organisms are still challenging. Nevertheless, combined application of metabolic engineering and system/synthetic biology tools for strain-design, construction and optimization may address the hurdles of inefficient production of flavonoids from microbes.

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Tables

Table 1. Selected examples of flavonoids of different classes.

Flavonoids	Selected Examples
Flavan	Tupichinol, kazinol Q, acutifolin A
Flavan-3-ol	Elephantorrhizol, guibourtinidol, epirobinetinidol, catechin
Flavan-4-ol	Apiforol, luteoforol, triphyllin A
Flavan-3,4-diol	Leucocyanidin, melacacidin, leucopaeonidin, leucofisetinidin
Flavanone	(2 <i>S</i>)-Naringenin, (2 <i>S</i>)-eriodictyol, (2 <i>S</i>)-pinocembrin, hesperetin
Dihydroflavonol	Diosalol, plumbaginol, floranol, dihydrokaempferol, dihydroquercetin
Flavone	Chrysin, baicalein, luteolin, velutin, apigenin
Flavonol	Kaempferol, fisetin, morin, rhamnetin, isorhamnetin, myrecetin, galangin, isorhamnetin, pachypodol
Anthocyanidin	Cyanidin, delphinidin, malvidin, rosinidin
Isoflavonoids	
Isoflavan	Equol
Isoflavanone	(+)-Sophorol, (+/-)-5-Deoxykievitone
Isoflavone	Daidzein, genistein, formononetin, biochanin A
Isoflav-3-ene	Burtinol-A, burtinol-B
Isoflav-2-ene	Glabrene, 2-methoxyjudaicin, hagin D
3-Arylcoumarin	Scandenin, glycycomarin
Rotenoid	Deguelin, tephrosin
Pterocarpin	Medicarpin, phaseolin
Coumestan	Coumestrol, wedelolactone, plicadin
Neoflavonoids	
Neoflavone	Calophyllolide, dalbergin
3,4-Dihydro-4-aryl coumarin	Calofloride
Neoflavene	Dalbergichromene
Other flavonoids	
Aurone	Aureusidin, leptosidin
Stilbene	Resveratrol, pinosylvin, pterostilbene, piceatannol
Chalcone	Phloretin, naringenin chalcone

Table 2. Therapeutic applications of selected flavonoids.

Compound	Therapeutic effects	References
Anticancer		
Kaempferol	Inhibition of activation of p53 mediated, mitochondrion-dependent apoptotic signaling.	Xiao et al., 2012
Quercetin	Modulation of PKC signaling and induction of apoptosis through suppression of ROS and TNFR1. Prevention of cancer metastasis in prostate cancer.	Bhat et al., 2014; Lee et al., 2015; Maurya and Vinayak, 2015
Apigenin	Inhibition of T-24 cell proliferation. Regulation of a p53-Bax-caspase-3 apoptotic pathway in neuroblastoma cells. Inhibition of STAT3 signaling in HER2-expression and block of cell proliferation by arresting different phases of cell cycle.	Ruela-de-Sousa et al., 2010; Seo et al., 2014; Torkin et al., 2005
Genistein	Reducing the incidence of carcinogen-induced adenocarcinoma and cancer progression suppressing tumor growth and metastatic transition. Triggers cell death via activation of caspase-3/9 reaction oxygen species increases significantly.	Hormann et al., 2012; Kim et al., 2014; Song et al., 2015
Resveratrol	Effects on cancer related transcription factors (p53), cell cycle regulation and induction of apoptosis (G2 and S phases), cell differentiation and proliferation (inhibition of mitogen-activated protein kinases, activator protein-1, nuclear factor (NF)κB)	Baur and Sinclair, 2006; Catalgol et al., 2012
Anti-inflammatory		
Hesperedin	Suppression of inflammatory response induced by <i>A. hydrophila</i> toxins through down modulation of reaction oxygen species production other factors stimulation.	Abuelsaad et al., 2014
Rhamnetin	Suppresses tumor necrosis factor (mTNF)-α, mouse macrophase inflammatory protein (mMIP)-1, and mMIP-2 cytokine production in lipopolysaccharide or Interferon (INF)-γ stimulated macrophages.	Jnawali et al., 2014
Fisetin	Apoptosis by up-regulation of p53 and down regulation of NF-κ B activity. Modulation of NF-κB pathway for regulation of inflammation and proliferation of tumor cells. Inhibits direct binding to mTOR and p70S6 kinase to inhibit cell proliferation.	Li et al., 2014; Sung et al., 2007; Syed et al., 2014
Afzelin	DNA-protective, antioxidant, and anti-inflammatory. Skin protection from UVB-induce damage.	Shin et al., 2013
Naringin	Block activity of NFκB, suppression of tumor NF-α and IL-8	Nie et al., 2012
Luteolin	Inhibit TNF-α production, NF-κB activation	
Kaempferol	Inhibit the production of COX-2, PGE2, and MMP-1 and MMP-3	
Genistein	Tyrosine kinase inhibitor, inhibition of NF-κB activation following AMP activated protein kinase stimulation connected with metabolic syndrome disease.	Ribeiro et al., 2015
Cardioprotective		
Hesperedin	Upregulation of protein levels of nuclear factor erythroid 2-related factor 2, which is responsible for antioxidant activity.	Elavarasan et al., 2012
Resveratrol	VSMC migration and proliferation, proliferation of pulmonary artery endothelial cells.	Vidavalur et al., 2006; Catalgol et al., 2012
Neuro-protective		
Resveratrol	Plaque formation, motor and cognitive functions, spinal cord injuries, amyloid-beta levels.	Catalgol et al., 2012
Naringenin	Down-regulation of NOD2, RIP2, NF-κB, MMP-9 and up-regulation of claudin-5 expression.	Bai et al., 2014
Alzheimer's disease		
Fisetin	Reduction of cyclin-dependent kinase 5 (Cdk5) activator p35 clavage product, p25, in control of Alzheimer's disease.	Currais et al., 2014

Silibinin	Dual inhibitor of acetylcholinesterase and B β aggregations down regulation.	Duan et al., 2015 Souza et al., 2015
Parkinson's disease		
7,8-dihydroxyflavone	TrkB provokes its dimerization and auto phosphorylation and activates downstream signaling cascade, induce BDNF-like behavioral phenotypes	Devi and Ohno, 2012
Anti-viral		
Apigenin	Inhibition of viral post entry stage of foot-and-mouth disease viral infection. Inhibition of hepatitis C virus replication by decreasing mature microRNA122 levels.	Shibata et al., 2014; Qian et al., 2015
Respiratory disease		
Hesperedin Kaempferol-3-O-rhamnoside	Hesperidin reduces the Th2 cytokines (IL-5), eotaxin, ovalbumin-specific IgE production, and eosinophil infiltration via inhibition of GATA-3 transcription factor.	Chung et al., 2015; Kim et al., 2011
Antidiabetic		
Kaempferitrin	Kaempferitrin activates insulin signaling pathway and stimulates secretion of adiponectin in 3T3-L1 adipocytes more than insulin itself.	Tzeng et al., 2009
Resveratrol	Obesity, insulin resistance, glucose homeostasis, polyphagia, polydipsia, Renal dysfunction.	Bai et al., 2014; Catalgol et al., 2012; Vidavalur et al. 2006
Antiaging		
Resveratrol	Inhibit expression of INK4 α in human dermal fibroblasts, down regulation of Ras-GRF1, RAC3 and UBE2D3	
Chrysin	Prevent age-associated memory loss by free radical scavenger action and modulation of BDNF of central nervous system.	Souza et al., 2015

Abbreviations: AMP: Adenosine monophosphate; BDNF: brain derived neurotrophic factor; COX-2:

cyclooxygenase; GATA3: trans-acting T-cell-specific transcription factor; GRF1: guanine nucleotide-releasing factor 1; HER2: human epidermal growth factor 2; IL: interleukin; INK: cyclin-dependent kinase; MMP: matrix metalloproteinase; mTOR: mechanistic target of rapamycin; NOD2: nucleotide-binding oligomerization domain-containing protein 2; PGE2: prostaglandin E2; PKC: protein kinase C; Ras-GRF1: Ras protein-specific guanine nucleotide-releasing factor 1; RAC3: Ras-related C3 botulinum toxin substrate 3; RIP2: receptor-interacting protein kinase 2; STAT3: signal transducer and activator of transcription 3; TNFR1: tumor necrosis factor receptor 1; UBE2D3: ubiquitin-conjugating enzyme E2 D3; VSMC: vascular smooth muscle cell.

Table 3. Production of flavonoids from microbial hosts.

Product	Precursor	Overexpressed Gene/Source	Platform Organism	Titer (mgL ⁻¹)	References
Naringenin	Tyrosine	<i>R. rubra</i> (PAL), <i>S. coelicolor</i>			
Pinocembrin	Phenylalanine	A3(2) (ScCCL), and <i>G. echinata</i> (CHS)	<i>E. coli</i>		Hwang et al., 2003
<i>p</i> -Coumaric acid	Phenylalanine	<i>P. trichocarpa</i> x <i>P. deltoides</i> (PAL, C4H, CPR)	<i>S. cerevisiae</i>		Ro and Douglas, 2004
Naringenin	Glucose	<i>R. capsulatus</i> (TAL), <i>A. thaliana</i> (4CL, CHS)	<i>E. coli</i>	20.8	Watts et al., 2004
Naringenin	Glucose	<i>R. toruloides</i> (TAL), <i>A. thaliana</i> (At4CL1), <i>H. androsaemum</i> (CHS)	<i>S. cerevisiae</i> / $\Delta pad1$	7.0 0.8	Jiang et al., 2005
Naringenin	Tyrosine	<i>R. rubra</i> (PAL), <i>S. coelicolor</i>		57	
Pinocembrin	Phenylalanine	A3(2) (ScCCL), <i>G. echinata</i> (CHS), <i>P. lobata</i> (PCHI), <i>C. glutamicum</i> (accBC and dtsR1)	<i>E. coli</i>	58	Miyahisa et al., 2005
Apigenin	Tyrosine	<i>R. rubra</i> (PAL), <i>S. coelicolor</i>		13	
Chrysin	Phenylalanine	A3(2) (ScCCL), <i>G. echinata</i> (CHS), <i>P. lobata</i> (CHI), <i>P. crispum</i> (F51) <i>C. glutamicum</i> (accBC and dtsR1)		9.4	
Kaempferol	Tyrosine	<i>R. rubra</i> (PAL), <i>S. coelicolor</i>	<i>E. coli</i>	15.1	Miyahisa et al., 2006
Galangin	Phenylalanine	A3(2) (ScCCL), <i>G. echinata</i> (CHS), <i>P. lobata</i> (CHI), <i>P. crispum</i> (FNS1), <i>Citrus</i> species (F3H, FLS), <i>C. glutamicum</i> (accBC and dtsR1)		1.1	
Resveratrol	Phenylalanine	<i>Populus trichocarpa</i> x <i>P.</i>		0.29	
	<i>p</i> -Coumaric acid	<i>deltoides</i> (PAL, CPR), <i>G. max</i> (C4H, 4CL), <i>V. vinifera</i> (RS)		0.31	
Naringenin	Phenylalanine	<i>P. trichocarpa</i> x <i>P. deltoides</i> (PAL, CPR), <i>G. max</i> (C4H, 4CL, CHS, CHI)		8.9	
	<i>p</i> -Coumaric acid			15.6	
Genistein	Phenylalanine	<i>P. trichocarpa</i> x <i>P. deltoides</i> (PAL, CPR), <i>G. max</i> (C4H, 4CL, CHS, CHI, IFS)	<i>S. cerevisiae</i>	0.1	
	<i>p</i> -Coumaric acid			0.14	Trantas et al., 2009
	Naringenin			7.7	
Kaempferol	Phenylalanine	<i>P. trichocarpa</i> x <i>P. deltoides</i> (PAL, CPR), <i>G. max</i> (C4H, 4CL, CHS, CHI, F3H), <i>S. tuberosum</i> (FLS)		1.3	
	<i>p</i> -Coumaric acid			0.9	
	Naringenin			4.6	
Quercetin	Phenylalanine	<i>P. trichocarpa</i> x <i>P. deltoides</i> (PAL, CPR), <i>G. max</i> (C4H, 4CL, CHS, CHI, F3H, F3'H), <i>S. tuberosum</i> (FLS)		-	
	<i>p</i> -Coumaric acid			0.26	
	Naringenin			0.38	
Naringenin	Glucose	<i>A. thaliana</i> (4CL3, CHS1, CHI1, codon optimized C4H and PAL1, codon optimized CPR variants (CPR1 or CPR2), codon optimized CHS3) <i>R. capsulatus</i> (codon optimized TAL1)	<i>S. cerevisiae</i> /Aro4::Aro4G226S/ Δ ARO3 Δ PDC1 Δ PDC5 Δ PDC6	112.9 (2-L ferment or)	Koopman et al. 2012

Eriodictyol	Glucose	<i>R. glutinis</i> (TAL), <i>P. crispum</i> (4CL), <i>P. X hybrid</i> (CHS), <i>M. sativa</i> (CHI), Fusion protein of F3'H from <i>G. hybrida</i> and CPR from <i>C. roseus</i> ; <i>C. glutamicum</i> ATCC 13032 (<i>accBC</i> , <i>dtsR1</i>), <i>E. coli</i> BL21(DE3) (<i>acs</i>)	<i>E. coli</i> /Δ <i>ackA</i>	5.70		
	L-tyrosine			107		Zhu et al., 2014
4-Coumaric acid	Glucose	<i>S. espanaensis</i> (codon optimized TAL and C3H), <i>A. thaliana</i> (codon optimized COM)	<i>E. coli</i> /Δ <i>tyrR</i> , <i>AroG::AroG^{fb}</i>	974		Kang et al., 2012
Caffeic acid			<i>tyrA::tyrA^{fb}</i>	150		
Ferulic acid				196		
3,5-dihydroxy 4'-methoxy stilbene	Glucose					
3,4'-dimethoxy -5-hydroxy stilbene		<i>S. espanaensis</i> (codon optimized TAL), <i>S. bicolor</i> (<i>sbOMT1</i> , <i>sbOMT3</i>), <i>A. hypogaea</i> (<i>AhSTS</i>)	<i>E. coli</i>	2.5		Kang et al., 2014
3,5,4'-trimethoxy stilbene		<i>S. coelicolor</i> A3(2) (<i>ScCCL</i>)		0.17		
Resveratrol-4'- <i>O</i> -glucoside	Glucose	<i>S. espanaensis</i> (codon optimized TAL), <i>A. hypogaea</i> (<i>STS</i>) <i>S. coelicolor</i> A3(2) (<i>ScCCL</i>), <i>B. licheniformis</i> DSM 13 (<i>YjiC</i>)	<i>E. coli</i>	0.05		
<i>trans</i> -Piceid				7.5		Choi et al., 2014
<i>cis</i> -Piceid				2.5		
Sakuranetin	Glucose	<i>S. espanaensis</i> (TAL), <i>O. sativa</i> (4CL), <i>Populus euramericana</i> (CHS), <i>O. sativa</i> (NOMT), <i>E. coli</i> (<i>ppsA</i> , <i>tktA</i> , <i>aroG^{fb}</i> , <i>tyrA^{fb}</i>)		1.7		
		<i>S. espanaensis</i> (TAL), <i>O. sativa</i> (4CL), <i>Populus euramericana</i> (CHS), <i>G. max</i> (<i>SOMT2</i>), <i>E. coli</i> (<i>ppsA</i> , <i>tktA</i> , <i>aroG^{fb}</i> , <i>tyrA^{fb}</i>)		40.1		
Ponciretin		<i>S. espanaensis</i> (TAL), <i>O. sativa</i> (4CL), <i>Populus euramericana</i> (CHS), <i>G. max</i> (<i>SOMT2</i>), <i>E. coli</i> (<i>ppsA</i> , <i>tktA</i> , <i>aroG^{fb}</i> , <i>tyrA^{fb}</i>)	<i>E. coli</i> /Δ <i>tyrR</i> Δ <i>pheA</i> Δ <i>icdA</i>	42.5		Kim et al., 2013a
Kaempferol 3- <i>O</i> -rhamnoside	Glucose	<i>S. espanaensis</i> (TAL), <i>O. sativa</i> (4CL), <i>Populus euramericana</i> (CHS), <i>P. euramericana</i> (FLS), <i>A. thaliana</i> (<i>AtUGT78D1</i>), <i>E. coli</i> (<i>ppsA</i> , <i>tktA</i> , <i>aroG^{fb}</i> , <i>tyrA^{fb}</i>)		57		Yang et al., 2014
Pinocembrin	Cinnamic acid	<i>P. crispum</i> (<i>Pc4CL2</i>), <i>P. hybrida</i> (<i>PhCHS</i>), <i>M. sativa</i> (<i>MsCHI</i>)	<i>E. coli</i>	429		Leonard et al., 2007
Naringenin	<i>p</i> -Coumaric acid	<i>P. luminescens</i> (<i>accABCD</i> , <i>BirA</i>), <i>E. coli</i> (<i>ackA</i> , <i>pta</i> , <i>acs</i>)		119		
Eriodictyol	Caffeic acid	<i>R. trifolii</i> (<i>matBC</i>), <i>P. crispum</i> (<i>Pc4CL2</i>), <i>P. hybrida</i> (<i>PhCHS</i>), <i>M. sativa</i> (<i>MsCHI</i>)		52		
Pinocembrin	Cinnamic acid	(Ceruleenin added)	<i>E. coli</i>	710		
Naringenin	<i>p</i> -Coumaric acid			186		
Eriodictyol	Caffeic acid			54		
Chrysin	Cinnamic acid	<i>R. trifolii</i> (<i>matBC</i>), <i>P. crispum</i> (4CL, <i>FSI</i>), <i>Petunia</i> (CHS), <i>M. sativa</i> (CHI)		5		Leonard et al., 2008
Apigenin	<i>p</i> -Coumaric acid	(Ceruleenin added)		110		
Luteolin	Caffeic acid			4		
Cyanidin 3- <i>O</i> -glucoside	Catechin	ANS-3-GT fusion protein, <i>E. coli</i> (<i>pyrE</i> , <i>pyrF</i> , <i>cmk</i> , <i>ndk</i> , <i>pgm</i> , <i>galU</i>)	<i>E. coli</i> /Δ <i>udg</i> Δ <i>galE</i>	104		
Pelargonidin			Δ <i>galT</i>	113		
3- <i>O</i> -glucoside	Afzelechin					
Pelargonidin	Naringenin	<i>M. domestica</i> (<i>MdF3H</i>), <i>A. andraeanum</i> (<i>DFR</i>), <i>P. hybrida</i> (<i>PhANS</i>), <i>A. thaliana</i> (<i>At3GT</i>),	<i>E. coli</i>	0.98		Yan et al., 2005
3- <i>O</i> -glucoside						
Cyanidin 3- <i>O</i> -	Eriodictyol					

glucoside		and <i>Desmodium uncinatum</i> (DuLAR)		2.07	
Pelargonidin 3-O-6''-O-malonylglucoside	Naringenin	<i>M. domestica</i> (MdF3H), <i>A. andraeanum</i> (DFR), <i>P. hybrida</i> (PhANS), <i>A. thaliana</i> (At3GT), and <i>Desmodium uncinatum</i> (DuLAR), <i>Dahlia variabilis</i> (Dv3MaT)		0.10	
Cyanidin 3-O-6''-O-malonylglucoside	Eriodictyol		<i>E. coli</i>	0.12	
Pelargonidin 3-O-glucoside	Afzelechin	Fusion of <i>P. hybrida</i> (PhANS) and <i>A. thaliana</i> (At3GT), <i>E. coli</i> (Pgm, galU)		78.9	
Cyanidin 3-O-glucoside	(+)-Catechin			70.7	
Resveratrol	<i>p</i> -Coumaric acid	<i>A. thaliana</i> (At4CL1), <i>V. vinifera</i> (VvSTS) <i>E. coli</i> (ACC/BirA) (Cerulenin added)	<i>E. coli</i> BW27784	2340	Lim et al., 2011
Naringenin	Glucose	<i>R. glutinis</i> (RgTAL), <i>P. crispus</i> (Pc4CL1), <i>P. hybrida</i> (PhCHS), <i>M. sativa</i> (MsCHI), <i>R. trifoli</i> (matBC) (Cerulenin added)	<i>E. coli</i>	84	Santos et al., 2011
(+)-Catechin	Eriodictyol	<i>C. sinensis</i> (CsF3H) <i>A. andraeanum</i> (AaFDR), <i>D. uncinatum</i> (DuLAR) (Scaffolding proteins)	<i>E. coli</i> Δ <i>pgi</i> Δ <i>ppc</i>	910.9	Zhao et al., 2015
(+)-Afzelechin	Naringenin	<i>C. sinensis</i> (CsF3H) <i>Fragaria ananassa</i> (FaDFR), <i>D. uncinatum</i> (DuLAR)	<i>E. coli</i>	39.5	
Resveratrol	Glucose	<i>R. sphaeroides</i> (Codon optimized RsTAL), fused protein of <i>A. thaliana</i> (At4CL) and <i>V. vinifera</i> (VvSTS)	<i>S. cerevisiae</i>	1.06	Wang et al., 2011b
	Tyrosine			1.90	
Cyanidin 3-O-glucoside	(+)-Catechin	<i>A. thaliana</i> (At3GT) fused with <i>P. hybrida</i> (PhANS) <i>E. coli</i> (Pgm, galU)	<i>E. coli</i>	70.7	Yan et al., 2008
Pelargonidin 3-O-glucoside	(+)-Afzelechin			78.9	
Cyanidin 3-O-glucoside	Naringenin	<i>M. domestica</i> (MdF3H, MdANS) <i>A. andraeanum</i> (AaDFR), <i>P. hybrida</i> (UGT3)	<i>E. coli</i>	0.0056	Yan et al., 2005
Pelargonidin 3-O-glucoside	Eriodictyol			0.006	
Kaempferol	<i>p</i> -Coumaric acid	<i>P. crispum</i> (Pc4CL-2), <i>A. thaliana</i> (FLS1), <i>P. x hybrida</i> (PhCHS, PhCHI), <i>M. domestica</i> (MdF3H), <i>C. roseus</i> (fused F3'5'H and CPR)		0.2	
Quercetin	Naringenin			0.9	
	<i>p</i> -Coumaric acid		<i>E. coli</i>	0.05	Leonard et al., 2006
	Eriodictyol			1.1	
Myricetin	Naringenin/ Eriodictyol			0.01	
Resveratrol	<i>p</i> -Coumaric acid	<i>A. thaliana</i> (At4CL1), <i>A. hypogaea</i> (AhSTS)	<i>E. coli</i>	104.5	Watts et al., 2006
Piceatannol	Caffeic acid	<i>P. crispum</i> (Pc4CL-2), <i>P. hybrida</i> (PhCHS), <i>M. sativa</i> (MsCHI), <i>A. thaliana</i> (AtF3H), <i>S. avermitilis</i> (SaOMT), <i>N. farcinica</i> (AccBC, birA, acs)		13.3	
7-O-methyl aromadendrin	<i>p</i> -Coumaric acid		<i>E. coli</i>	2.7	Malla et al., 2012
Naringenin		<i>S. coelicolor</i> (ScCCL), <i>A. thaliana</i> (codon optimized AtCHS)	<i>S. venezuelae</i>	4	Park et al., 2009
Pinocembrin	<i>p</i> -Coumaric acid			6	

Resveratrol	<i>p</i> -Coumaric acid	<i>A. hypogaea</i> (codon optimized		0.4	
Pinosylvin	Cinnamic acid	<i>AhSTS</i>), <i>S. coelicolor</i> (<i>ScCCL</i>)		0.6	
Kaempferol	Naringenin	<i>C. siensis</i> (codon optimized <i>F3H</i>),		nd	Park et al., 2010
Galanin	Pinocembrin	<i>C. unshius</i> (<i>FLS</i>)			
Naringenin	<i>p</i> -Coumaric acid	<i>S. coelicolor</i> (<i>ScCCL</i>), <i>A. thaliana</i>	<i>S.</i>	35.6	
Eriodictyol	Cinnamic acid	(codon optimized <i>AtCHS</i> , <i>AtCHI</i>)	<i>venezuelae</i>	44.1	
		<i>S. coelicolor</i> (<i>ScCCL</i>), <i>A. thaliana</i>	(<i>matBC</i>		Park et al., 2011
Chrysin	<i>p</i> -Coumaric acid	(codon optimized <i>AtCHS</i> , <i>AtCHI</i>),	from <i>S.</i>	30.9	
Apigenin	Cinnamic acid	<i>P. crispum</i> (codon optimized	<i>coelicolor</i>	15.3	
		<i>PcFSI</i>)	integrated)		
		<i>P. crispum</i> (<i>Pc4CL2</i>), <i>P. hybrida</i>	<i>E. coli</i> /		
Naringenin	<i>p</i> -Coumaric acid	(<i>PhCHS</i>), <i>M. sativa</i> (<i>MsCHI</i>)	<i>ΔsdhAΔ</i>	270	Fowler et al., 2009
Eriodictyol		<i>E. coli</i> (<i>coaA</i> , <i>acs</i>), <i>P. luminescens</i>	<i>adhEΔbrn</i>	150	
		(<i>ACC</i> , <i>BirA</i>)	<i>QΔcitE</i>		
Leudocyanidin	Dihydro-	<i>D. uncinatum</i> (<i>DuLAR</i>)	<i>E.</i>	817	
(+)-Catechin	quercetin	<i>D. uncinatum</i> (<i>DuLAR</i>) <i>A.</i>	<i>coli/Δpgi</i>	38.8	Chemler et al., 2010a
		<i>andraeanum</i> (<i>AaDFR</i>)	<i>ΔpldAΔppc</i>		
		<i>P. crispum</i> (<i>Pc4CL2</i>), <i>P. hybrida</i>			
		(<i>PhCHS</i>), <i>M. sativa</i> (<i>MsCHI</i>)			
Naringenin		<i>E. coli</i> (<i>accABCD</i> , <i>pgk</i> , <i>aceEF</i> ,	<i>E. coli</i> /	474.2	Xu et al., 2011
	<i>p</i> -Coumaric acid	<i>lpdA</i>)	<i>ΔfumC</i>		
		<i>A. thaliana</i> (<i>At4CL2</i>) <i>V. vinifera</i>	<i>ΔsucC</i>		
Resveratrol		(<i>VvSTS</i>) <i>E. coli</i> (<i>accABCD</i> , <i>pgk</i> ,		1600	Bhan et al., 2013
		<i>aceEF</i> , <i>lpdA</i>)			
		<i>E. coli</i> (<i>aroF</i> , <i>pheA^{fbr}</i>), <i>R. glutinis</i>			
(2 <i>S</i>)-	Glucose	(<i>RgPAL</i>), <i>P. crispum</i> (<i>Pc4CL</i>), <i>P.</i>	<i>E. coli</i>	40.02	Wu et al., 2013a
Pinocembrin		<i>X hybrida</i> (<i>PhCHS</i>), <i>M. sativa</i>			
		(<i>MsCHI</i>), <i>R. trifolii</i> (<i>matBC</i>)			
		<i>E. coli</i> (<i>aroG^{fbr}</i> , <i>tyrA^{fbr}</i>), <i>R.</i>			
		<i>glutinis</i> (<i>RgTAL</i>), <i>P. crispum</i>			
(2 <i>S</i>)-	Glucose	(<i>Pc4CL</i>), <i>P. X hybrida</i> (<i>PhCHS</i>),	<i>E. coli</i>	100.64	Wu et al., 2014b
Naringenin		<i>M. sativa</i> (<i>MsCHI</i>), <i>R. trifolii</i>			
		(<i>matBC</i>)			
		<i>R. glutinis</i> (<i>RgTAL</i>), <i>V. vinifera</i>			
Resveratrol	L-Tyrosine	(<i>VvSTS</i>), <i>R. trifolii</i> (<i>matBC</i>)	<i>E. coli</i>	35.02	Wu et al., 2013b
		<i>P. crispum</i> (<i>Pc4CL2</i>), <i>P. X</i>			
		<i>hybrida</i> (<i>PhCHS</i>), <i>M. sativa</i>		36.7	
		(<i>MsCHI</i>)			
Naringenin	<i>p</i> -Coumaric acid	<i>P. crispum</i> (<i>Pc4CL</i>), <i>P. X hybrida</i>	<i>E. coli</i>		Xu et al., 2012
		(<i>PhCHS</i>), <i>M. sativa</i> (<i>MsCHI</i>) <i>P.</i>		80	
		<i>luminescens</i> (<i>accABCD</i>)			
(-)-				0.01	
Epicatechin					
(-)-					
Epicatechin	Eriodictyol	<i>C. sinensis</i> (<i>F3H</i> , <i>DFR</i> , <i>LCR</i>)	<i>E. coli</i>	0.36	Umar et al., 2012
gallate					
(-)-Catechin				0.13	
hydrate					
(-)-Catechin				0.04	
gallate					
		<i>M. sativa</i> (<i>MsCHI</i>), <i>P. hybrid</i>			
Naringenin		(<i>PhCHS</i>), <i>P. crispum</i> (<i>Pc4CL2</i>)	<i>E. coli</i>	91.31	
	<i>p</i> -Coumaric acid	<i>P. crispum</i> (<i>Pc4CL2</i>) <i>V. vinifera</i>	(<i>fabD</i>		Yang et al., 2015a
Resveratrol		(<i>VvSTS</i>)	inhibited)	268.2	
		(<i>fabD</i> inhibited)			

Glycosylation in <i>E. coli</i>					
Naringenin-7- <i>O</i> -xyloside	Naringenin	<i>M. echinospora</i> (calS8, calS9), <i>A. thaliana</i> (ArGT-4)	<i>E. coli</i> /Δ <i>pgi galU</i> integrated	nd	Simkhada et al., 2009
Quercetin 3- <i>O</i> -xyloside	Quercetin	<i>N. farcinica</i> (nfa44530), <i>E. coli</i> (galU), <i>M. echinospora</i> (calS89), <i>A. thaliana</i> (ArGT-3)	<i>E. coli</i> /Δ <i>pgi Δzwf ΔushA</i>	42.5	Pandey et al., 2013b
Quercetin 3- <i>O</i> -xyloside	Quercetin	<i>A. thaliana</i> (uxs, AtUGT78D3 H380Q) <i>E. coli</i> (ugd)	<i>E. coli</i> /Δ <i>arnA</i>	150	Han et al., 2014
Kaempferol 3- <i>O</i> -rhamnoside	Kaempferol	<i>T. caldophilus</i> (tgs), <i>S. typhimurium</i> LT2 (dh), <i>S. antibioticus</i> Tu99 (ep, kr), <i>A. thaliana</i> (ArGT-3)		12.9	
Quercetin 3- <i>O</i> -rhamnoside	Quercetin	<i>T. caldophilus</i> (tgs), <i>S. typhimurium</i> LT2 (dh), <i>Streptomyces</i> sp KCTC0041BP (gerFK), <i>A. thaliana</i> (ArGT-3)	<i>E. coli</i> /Δ <i>pgi</i>	24	Simkhada et al., 2010
Quercetin 3- <i>O</i> -alloside	Quercetin			Trace	
Quercetin-3- <i>O</i> -4-deoxy-4-formamido-L-arabinose	Quercetin	<i>A. thaliana</i> (AtUGT78D3) <i>E. coli</i> (ugd, arnA, arnB)	<i>E. coli</i>	70	Kim et al., 2010a
Quercetin 3- <i>O</i> -arabinoside	Quercetin	<i>A. thaliana</i> (uxs, AtUGT78D3) <i>E. coli</i> (ugd), <i>Oryza sativa</i> (uxe)	<i>E. coli</i> /Δ <i>arnA</i>	158	Han et al., 2014
Quercetin 3- <i>O</i> - <i>N</i> -acetyl glucosamine	Quercetin	<i>A. thaliana</i> (AtUGT78D2)	<i>E. coli</i> /Δ <i>galU</i>	380	Kim et al., 2012b
Quercetin 3- <i>O</i> -6-deoxytalose	Quercetin	<i>Actinobacillus actinomycetemcomitans</i> (tll), <i>A. thaliana</i> (AtUGT78D1)	<i>E. coli</i> /Δ <i>galU ΔrffA ΔrfbD</i>	98	Yoon et al., 2012
Quercetin 3- <i>O</i> -glucoside-7- <i>O</i> -rhamnoside	Quercetin 3- <i>O</i> -glucoside	<i>A. thaliana</i> (AtRHM, UGT89C1)	<i>E. coli</i>	20	Roepke and Bozzo, 2013
Myricetin 3- <i>O</i> -rhamnoside	Myricetin	<i>T. caldophilus</i> (tgs), <i>S. typhimurium</i> LT2 (dh), <i>S. antibioticus</i> Tu99 (ep, kr), <i>A. thaliana</i> (ArGT-3)	<i>E. coli</i> /Δ <i>pgi Δzwf</i>	25.8	Thuan et al., 2013a
Apigenin-7- <i>O</i> -glucoside	Apigenin	<i>A. thaliana</i> (AGT), <i>E. coli</i> (galU)	<i>E. coli</i> /Δ <i>pgi</i>	39.28	Thuan et al., 2013b
Baicalein-7- <i>O</i> -glucoside	Baicalin		Δ <i>zwf</i> Δ <i>ushA</i>	33.19	
Kaempferol 3- <i>O</i> -glucoside	Naringenin	<i>A. thaliana</i> (AtF3H, AtFLS1), <i>N. farcinica</i> (nfa44530), <i>E. coli</i> (galU), <i>G. max</i> (UGT78K1)	<i>E. coli</i> /Δ <i>pgi Δzwf ΔushA</i>	109.3	Malla et al., 2013
Genistin	Genistein	<i>E. coli</i> (galU), <i>G. max</i> (GmIF7GT, GmIF7MaT)	<i>E. coli</i> /Δ <i>pgi</i>	75.9	Koirala et al., 2014a
Daidzin	Daidzein		Δ <i>zwf</i> Δ <i>ushA</i>	67.65	
Genistin-6"- <i>O</i> -malonate	Genistein	<i>N. farcinica</i> (nfa9890, nfa9940, nfa9950, nfa3550), <i>G. max</i> (GmIF7GT, GmIF7MaT)	<i>E. coli</i> /Δ <i>zwf</i>	7.37	
Daidzin-6"- <i>O</i> -malonate	Daidzein			6.23	

Genistin	Genistein					
Genistein 4'- O-glucoside						
Genistein 4',7- O-diglucoside						
Daidzin	Daidzein	<i>N. farcinica</i> (nfa44530), <i>E. coli</i> (galU), <i>B. licheniformis</i> DSM13 (YjiC)	<i>E. coli</i> /Δpgi Δzwf ΔushA	nd		Pandey et al., 2014a
Daidzein 4'-O- glucoside						
Daidzein 4',7- O-diglucoside						
Ononin	Formononetin					
Sissotrin	Biochanin A					
Quercetin 3- O-galactoside	Quercetin	<i>P. hybrida</i> (PhUGT), <i>O. sativa</i> (uge)	<i>E. coli</i>	280		
Quercetin 3- O-glucuronide	Quercetin	<i>Antirrhinum majus</i> (AmUGT10) or <i>V. vinifera</i> (VvUGT)	<i>E. coli</i> /ΔaraA	687		Kim et al., 2015b
Luteolin 7-O- glucuronide	Luteolin	<i>E. coli</i> (ugd)		300		
Fisetin 3-O- glucoside	Fisetin	<i>Zymomonas mobilis</i> (glf, glk), <i>E. coli</i> (pgm2, galU) <i>G. max</i> (UGT78K1)		392.6		
Fisetin 3-O- rhamnoside	Fisetin	<i>Zymomonas mobilis</i> (glf, glk), <i>E. coli</i> (pgm2), <i>T. caldophilus</i> (tgs), <i>S. typhimurium</i> LT2 (dh), <i>S. antibioticus</i> Tu99 (ep, kr), <i>A. thaliana</i> (ArGt-3)	<i>E. coli</i>	342		Parajuli et al., 2015
Quercetin 3- O-4,6 dideoxy 4-amino-D- galactoside	Quercetin	<i>T. caldophilus</i> (tgs), <i>S. typhimurium</i> LT2 (dh), <i>E. coli</i> (WecE), <i>A. thaliana</i> (ArGt-3)				
Kaempferol 3- O-4,6 dideoxy 4-amino -D- galactoside	Kaempferol		<i>E. coli</i> /Δpgi Δzwf ΔgalU	nd		Pandey et al., 2015
Quercetin 3- O-3,6 dideoxy 3-amino-D- galactoside	Quercetin	<i>T. caldophilus</i> (tgs), <i>S. typhimurium</i> LT2 (dh), <i>Aneurinibacillus thermoaerophilus</i> L420-91 ^T (codon optimized <i>fdtA</i> , <i>fdtB</i>), <i>A. thaliana</i> (ArGt-3)				
Kaempferol 3- O-3,6 dideoxy 3-amino-D- galactoside	Kaempferol					
Glucosides from <i>E. coli</i> harboring a glycosyltransferase						
Kaempferol 3- O-glucoside	Kaempferol	<i>O. sativa</i> (RF5)	<i>E. coli</i>	44.8		Kim et al., 2006c
Quercetin 3- O-glucoside	Quercetin			46.4		
Luteolin-4'- glucoside				10.86		
Luteolin 7-O- glucoside	Luteolin	<i>M. truncatula</i> (UGT73C8)	<i>E. coli</i>	6.52		He et al., 2008
Genistein 7-O- glucoside	Genistein			20		
Biochanin A7- O-glucoside	Biochanin A	<i>M. truncatula</i> (UGT71G1)	<i>E. coli</i>	13		
Isorhamnetin	Isorhamnetin	<i>P. trichocarpa</i> x <i>P. deltoides</i>	<i>E. coli</i>	39.7		Kim et al.,

3- <i>O</i> -glucoside Phloretin 4'- <i>O</i> -glucoside		(PGT-3)		60.1	2010b
Phloretin 2'- <i>O</i> -glucoside				9.59	
Phloretin 4,4'- <i>O</i> -diglucoside	Phloretin	<i>B. licheniformis</i> DSM 13 (YjiC)	<i>E. coli</i> /Δ <i>pgi</i> Δ <i>zwf</i> Δ <i>ushA</i>	35.96	Pandey et al., 2013a
Phloretin 2',4',4'- <i>O</i> - triglucoside				162.8	
Methylation using <i>E. coli</i>					
7-methoxy derivatives	Apigenin Kaempferol Luteolin Quercetin	<i>P. trichocarpa</i> x <i>P. deltoides</i> (POMT-7)	<i>E. coli</i>	17.9 17.4 16.8 19.9	Kim et al., 2006a
7-hydroxy-8- methoxyflavo- ne	7,8-dihydroxy- flavone	<i>S. peucetius</i> (SpOMT2884)	<i>E. coli</i>	52.57	Koirala et al., 2014b
3'-methoxy- quercetin 3',4'- dimethoxy quercetin 3'-methoxy-; 3',4'- dimethyl-; 7,3'- dimethyl- ; and 7, 3',4'- trimethyl- luteolin 3'-methyl-; 3',4'-dimethyl; 7,3',-dimethyl- ; and 7,3',4'- trimethyl- quercetin	Quercetin	Rice (ROMT-9) and (SOMT-2)	<i>E. coli</i>	nd 30.3	Kim et al., 2005b
7-methoxy derivatives	Luteolin	Poplar (POMT-7) and Rice (ROMT-9)	<i>E. coli</i>	nd	Kim et al., 2008
	Quercetin				
	Daidzein Genistein Kaempferol Quercetin Isorhamnetin Apigenin Naringenin	<i>S. avermitilis</i> MA-4680 (SaOMT-2)	<i>E. coli</i>	nd nd nd nd nd 16.3	Kim et al., 2006b
Acacetin Ponciretin 4'-methoxy quercetin	Apigenin Naringenin Quercetin	<i>G. max</i> (SOMT-2)	<i>E. coli</i>	nd 42.9	Kim et al., 2005a
Formononetin Biochanin A	Daidzein Genistein			nd nd	
Hydroxylation					
3',4', 7- trihydroxy isoflavone	Daidein		<i>S. avermitilis</i> MA-4680	21.6	Roh et al. 2009a
3',4',5,7- tetrahydro- xyisoflavone	Genistein	-		nd	

3',4',7-trihydroxy isoflavone	Daidzein	<i>N. farcinica</i> (CYP450- <i>nfa12130</i> with redox partner (<i>nfa12140-nfa12150</i>))	<i>E. coli</i>	nd	Choi et al., 2009
4',7,8-trihydroxy isoflavone		<i>N. farcinica</i> (CYP450- <i>nfa33880</i> with redox partners (<i>nfa33870-nfa33860</i>))			
3',4',7-trihydroxy isoflavone		<i>B. subtilis</i> 168 (CYP107H1) and <i>P. putida</i> putiaredoxin reductase (<i>camA</i>) and putodaredoxin (<i>camB</i>)	<i>E. coli</i>	trace	Roh et al., 2009b
Prenylation					
8-dimethylallyl naringenin	Naringenin	<i>Sophora flavescens</i> (SfN8DT-1)	<i>S. cerevisiae</i>	0.56	Sasaki et al., 2009

Abbreviations: *fbr*: feed-back inhibition resistance; nd: not determined

Note: The table contents are arranged according to the flow the manuscript content.

Table 4. Selected flavonoids modifying enzymes of different origin with their substrates promiscuity and features of modifications.

Post-modification enzymes	Sources	Substrates	Features	References
Glycosyltransferases				
ArGt-3	<i>A. thaliana</i>	Flavonols	3- <i>O</i> -rhamnose 3- <i>O</i> -xylose 3- <i>O</i> -allose 3- <i>O</i> -aminodeoxysugars	Simkhada et al., 2009, 2010; Thuan et al., 2013a; Pandey et al., 2013, 2015; Parajuli et al., 2015
UGT78D3		Flavonols	3- <i>O</i> -4-deoxy-4-formamido-L-arabinose 3- <i>O</i> -arabinose 3- <i>O</i> -xylose	Han et al., 2014 Kim et al., 2010a
UGT78D1		Quercetin Flavonols	3- <i>O</i> -6-deoxytaloside 3- <i>O</i> -rhamnose 3- <i>O</i> -glucose	Yoon et al., 2012 Kim et al., 2012a; Kim et al., 2013b; Ren et al., 2012
UGT89C1		Flavonols, Flavanone, Flavones Flavonol 3- <i>O</i> -glycosides	7- <i>O</i> -rhamnose	Yonekura-Sakakibara et al., 2007; Roepke and Bozzo, 2013; Parajuli et al., 2015
UGT78D2		Flavonols	3- <i>O</i> -glucose	Kim et al., 2013b
AGT		Apigenin Baicalein	7- <i>O</i> -glucoside	Thuan et al., 2013b
UGT89B1		Quercetin	Multiple <i>O</i> -monoglucosides 3,7-di- <i>O</i> -glucoside 7 and 3'-monoglucosides	Lim et al., 2004
UGT71C1			7,3'-di- <i>O</i> -glucoside	
UGT76E12			3 and 7- <i>O</i> -glucoside	
UGT74F1			3,7-di- <i>O</i> -glucoside 7 and 4'- <i>O</i> -glucoside	
YjiC	<i>Bacillus licheniformis</i> DSM13	Flavonoids, Isoflavonoids, Chalcones, Stillbenoids	Multiple <i>O</i> -glucosides Non-regiospecific Multiple NDP-sugars	Pandey et al., 2013ac; 2014ab; Gurung et al., 2013
AmUGT10/ VvUGT	<i>Antirrhinum majus</i> / <i>Vitis vinifera</i>	Quercetin Luteolin	Quercetin 3- <i>O</i> -glucuronide Luteolin 7- <i>O</i> -glucuronide	Kim et al., 2015b
PhUGT	<i>Petunia hybrid</i>	Quercetin	3- <i>O</i> -galactoside	
RF5	<i>Oryza sativa</i>	Quercetin Kaempferol Isorhamnetin	3- <i>O</i> -glucoside	Kim et al., 2006c
UGT71G1	<i>Medicago truncatula</i>	Genistein Biochanin A	7- <i>O</i> -glucoside	He et al., 2008
UGT73C8		Luteolin	4' and 7- <i>O</i> -glucosides	

BcGT1	<i>B. cereus</i> ATCC 10987	Flavonoids, Isoflavonoids	3- <i>O</i> -glucoside (preferred) Mono and di-glucosides	Ko et al., 2006 Ahn et al., 2009
XcGT-2	<i>X. campestris</i> pv. <i>campestris</i> ATCC 33913	Flavonoids with double bond between C-2 and C-3 and hydroxyl group at C-3' and C-4'	<i>O</i> -glycosylation, Monoglucoside, Glycosylation at C-3' position	Kim et al., 2007
OleD and its variants (OleD ^{PSA})	<i>S. antibioticus</i>	Flavonoids Isoflavonoids, Chalcones	<i>O</i> -glycosylation	Choi et al., 2012
Methyltransferases				
SOMT-2	<i>G. max</i>	Flavonoids, Isoflavonoids	4'- <i>O</i> -methylation	Kim et al., 2005a
POMT7	Poplar	Flavonoids	7- <i>O</i> -methylation	Kim et al., 2006a
ROMT-9	Rice	Flavonoids	3'- <i>O</i> -methylation	Kim et al., 2006d
SaOMT-2	<i>S. avermitilis</i> MA-4680	Flavonoids	7- <i>O</i> -methylation	Kim et al., 2006b
SpOMT-2884	<i>S. peuceitius</i> ATCC 27952	Isoflavonoids	<i>O</i> -methylation	Koirala et al., 2014b
CYPs				
Nfa 12130	<i>N. farcinica</i> IFM 10152	Daidzein	A-ring ortho-specific monohydroxylation	Choi et al., 2009
Nfa33880				
CYP107H1	<i>B. subtilis</i> 168	Daidzein	3'-ortho hydroxylation	Roh et al., 2009b
SAV2377	<i>S. avermitilis</i> MA4680	Genistein		
SAV5841		Chrysin,	B-ring hydroxylation	Pandey et al., 2011
SAV4539		Apigenin		
Prenyltransferases				
Orf2	<i>Streptomyces</i> sp. strain CL190	Flavonoids Isoflavonoids	Geranylation	Kuzuyama et al., 2005
SCO7190	<i>S. coelicolor</i> A3(2)	Flavonoids	Prenylation	Kumano et al., 2008
NovQ	<i>S. niveus</i>	Flavonoids	B-ring prenylation	Ozaki et al., 2009
SfN8DT-1	<i>Sophora flavescens</i>	Naringenin	8-prenylation	Sasaki et al., 2009

Figure Legends

Figure 1. Structures of different subclasses of flavonoids, isoflavonoids, neoflavonoids, and other minor flavonoids. Colors are used to differentiate each compound from another.

Figure 2. Biosynthesis pathway network of flavonoids in plants. Phenylalanine and tyrosine are produced via shikimate pathway in plastid. PAL and TAL convert L-phenylalanine and L-tyrosine to (*E*)-cinnamic acid and *p*-coumaric acid respectively. C4H and CPR also act on (*E*)-cinnamic acid to *p*-coumaric acid. 4CL activates phenylpropanoic acids to respective phenylpropanoyl-CoAs which enters to cytosol and undergoes condensation with three molecules of malonyl-CoA to produce naringenin chalcone. CHI further converts naringenin chalcone to naringenin, one of the major intermediates of flavones, flavonols, anthocyanidins, isoflavonoids, phlobaphenes by the action of various enzymes. Abbreviations of enzymes: PAL: phenyl ammonia lyase; TAL: tyrosine ammonia lyase; C4H: cinnamic acid 4-hydroxylase; CPR: cytochrome P450 reductase; 4CL: *p*-coumaroyl-CoA ligase; STS: stilbene synthase; CHS: chalcone synthase; CHI: chalcone isomerase; FS: flavone synthase; DFR: dihydroflavonol reductase; FLS: flavonol synthase; IFS: isoflavone synthase; and ANS: anthocyanidin synthase

Figure 3. Engineering approaches used for the heterologous production of flavonoids from different microbes. The sources of genes for flavonoid backbone synthesis could be plant, yeast or actinomycetes which is assembled in the platform organisms like *E. coli*, *S. cerevisiae*, or *S. venezuelae* using various approaches.

Figure 4. Biosynthesis of flavonoids by recombinant microbes. Flavonoid metabolic pathways connecting with malonyl-CoA generating pathways are presented at the bottom part of the figure. Red arrow and font colored genes are target genes for deletion while pink colored are inhibited/inactivated. Green colored arrows and genes encode for precursors and co-factors. Gene names are abbreviated as follows: *tyrR*: phenylalanine DNA-binding transcription repressor; *DAHPS*: 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase; *CM/PheA*: chorismate mutase/prephenate dehydratase; *PDT*: PDH: pyruvate dehydrogenase complex; *TAL*: tyrosine ammonia lyase; *4CL*: *p*-Coumaroyl-CoA ligase; *PAL*: phenyl ammonia lyase; *C4H*: cinnamic acid 4-hydroxylase; *CPR*: cytochrome P450 reductase; *CHS*: chalcone synthase; *CHI*: chalcone isomerase; *FSI*: flavone synthase I; *FSII*: flavone synthase II; *IFS*: Isoflavone synthase; *SOMT*: soybean *O*-methyltransferase; *NOMT*: *O. sativa O*-methyltransferase; *HID*: 2-hydroxyisoflavanone dehydratase; *F3H/FHT*: flavanone 3 β -hydroxylase; *DFR*: dihydroflavonol 4-reductase; *LAR*: leucocyanidin reductase; *ANS*: anthocyanin synthase; *3GT*: flavonoid 3-*O*-glycosyltransferase; *3MaT*: malonyl-CoA: anthocyanidin 3-*O*-glucoside-6''-*O*-malonyltransferase) *SaOMT-2*: *S. avermitilis* 7-*O*-methyltransferase; *FLS*: flavonol synthase; *F3'H*: flavonol 3'-hydroxylase; *F3'5'H*: flavonoid 3',5'-hydroxylase; *UGT78D1*: 3-*O*-glycosyltransferase; *UGT78K1*: 3-*O*-glucosyltransferase *STS*: stilbene synthase; *SbOMT1*: *S. bicolor O*-methyltransferase 1; *sbOMT3*: *S. bicolor O*-methyltransferase 3; *pyrE*: orotate phosphoribosyltransferase; *pyrF*: orotidine-5'-phosphate decarboxylase; *cmk*: cytidylate kinase; *ndk*: nucleoside diphosphate kinase; *galT*: galactose-1-phosphate uridylyltransferase; *galE*: UDP-galactose 4-epimerase; *pgi*: glucose-6-phosphate isomerase; *zwf*: glucose 6-phosphate dehydrogenase; *ushA*: UDP-glucose hydrolase; *pgm*: phospho-glucomutase; *galU*: glucose-1-phosphate uridylyltransdferase; *matB*: malonyl-CoA

synthetase; *matC*: dicarboxylate carrier protein; *coaA*: pantothenate kinase; *birA*: biotin ligase *accACD*: acetyl-CoA carboxylase complex encoding genes; *aceEF*: PDH complex encoding genes; *ACS*: acetyl-CoA synthase; *brnQ*: amino acid transporter; *sucCD*: succinyl-CoA synthetase; *sdhABCD*:succinate dehydrogenase; *fumC*: fumerase; *ppc*: phosphoenolpyruvate carboxylase; *citE*: citrate lyase; *pta*: phosphotransacetylase *ackA*:acetate kinase; *icdA*:isocitrate dehydrogenase; *fabB*: β -ketoacyl-acyl carrier protein synthase I; *fabF*: β -ketoacyl-acyl carrier protein synthase II; *fabD*: malonyl-CoA: ACP transacylase

Figure 5. Biosynthesis of natural and unnatural flavonoids, isoflavonoids, and stilbenes using various starting precursors. A) Precursor directed synthesis of chalcones, flavanones, flavones, flavonols, and stilbenes using recombinant *E. coli* supplemented with 20 different natural/unnatural carboxylic acid precursors. B) Precursor directed synthesis of natural/unnatural isoflavone derivatives from chemically modified flavanones in recombinant *S. cerevisiae*. Genes used for cell engineering are described in the text.

Figure 6. Biosynthetic pathways of different NDP-sugars reconstituted in *E. coli* for the production of natural/un-natural flavonoid glycosides. Glucose-1-phosphate is the branch point for the biosynthesis of TDP-sugars and UDP-sugars. Thymidyltransferase (*tgs*) transfers TDP from TTP whereas uridylyltransferase (*galU*) transfers UDP to glucose-1-phosphate to produce TDP- α -D-glucose and UDP- α -D-glucose, respectively. Further modifications of TDP- α -D-glucose and UDP- α -D-glucose by diverse enzymes result in production of various NDP-sugars. Yellow arrows represent reactions leading to diverse flavonoid glycoside structures produced using intracellular pool of NDP-sugars. Highlighted in green are the sugar units. Highlighted in red are deletion gene targets and in blue

overexpression gene targets for production of various NDP-sugars. Gene abbreviations are as follows: *glf*: glucose facilitator diffusion protein; *glk*: glucokinase; *pgm*: phosphoglucomutase; *tgs/rfbA*: glucose 1-phosphate thymidyltransferase; *dh/rfbB*: TDP-glucose 4, 6-dehydratase; *fdtA*: dTDP-6-deoxy-D-hex-4-ulose isomerase; *fdtB*: TDP-6-deoxy-D-galactose 3-aminotransferase *gerF*: *gerK1*: *epi/rfbC*: TDP-4-keto-6-deoxyglucose 3, 5-epimerase; *gerB*: TDP-6-deoxy-D-glucose 4-aminotransferase; *wecE*: TDP-6-deoxy-D-galactose 4-aminotransferase *kr/rfbD*: dTDP-4-dehydrorahmnose reductase; *tll*: dTDP-6-deoxy-L-lyxo-4-hexulose reductase; *rffA*: dTDP-4-oxo-6-deoxy-D-glucose transaminase; *galU*: glucose 1-phosphate uridylyltransferase *ushA*: UDP- -D-glucose hydrolase; *uge*: UDP-glucose epimerase; *calS8/ugd*: UDP-glucose 6-dehydrogenase; *calS9*: UDP-glucuronic acid decarboxylase; *uxs*: UDP-xylose synthase; *uxe*: UDP-xylose epimerase; *arnA*: UDP-4-amino-4-deoxy-L-arabinose (L-Ara4N) formyltransferase/ UDP-glucuronic acid C-4"-decarboxylase; *arnB*: UDP-L-Ara4-O-C-4" transaminase; *pgi*: glucose phosphate isomerase; *zwf*: D-glucose-6-phosphate dehydrogenase; *glmS*: L-glutamine:D-fructose-6-phosphate aminotransferase; *glmM*: phosphoglucosamine mutase; *glmU*: glucosamine-1-phosphate acetyltransferase; *ArGT-3*: flavonol 3-O-glycosyltransferase; *AtUGT78D1*: *UGT78K1*: flavonol 3-O-glucosyltransferase; *AtUGT78D3*: flavonol 3-O-glycosyltransferase; *VvUGT*: flavonoid UDP-glucuronosyltransferase; *PhUGT*: flavonoid UDP-galactosyltransferase; *AtUGT78D2*: flavonol 3-O-glycosyltransferase

Figure 7. Structures of glucosides of flavonoids produced after biotransformation using *E. coli* cells harboring GT. Blue colored GTs were used as enzymes for production of target glucosides. The details of genes used are explained in the text.

Figure 8. Biosynthesis of methylated, hydroxylated and prenylated flavonoid derivatives

from microbial cells. Genes used for the target modification of flavonoids are presented with blue color. The source and function of each gene is explained in text.

A)

B)