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Dudnik, Alexey; Gaspar, Paula; Neves, Ana Rute; Forster, Jochen

Published in:
Current Pharmaceutical Design

Link to article, DOI:
10.2174/1381612824666180515152049

Publication date:
2018

Document Version
Peer reviewed version

TITLE

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RUNNING TITLE

Microbial production of polyphenols

AUTHORS

Alexey Dudnik[1,#,*], Paula Gaspar[1,3,#], Ana Rute Neves[2] and Jochen Forster[1]

[1] Applied Metabolic Engineering Group, The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kemitorvet, Building 220, DK-2800, Kgs. Lyngby, Denmark; [2]Chr. Hansen A/S, Bøge Allé 10-12, DK-2970, Hørsholm, Denmark.

*Corresponding author:
Alexey Dudnik
Email: adud@biosustain.dtu.dk
Phone: + 45 93 51 11 01
Fax: +45 45 25 80 01

[3]Current address: Chr. Hansen A/S, Bøge Allé 10-12, DK-2970, Hørsholm, Denmark.

[#]These authors contributed equally to this work.

Keywords: *Escherichia coli*, fisetin, metabolic engineering, microbial cell factories, polyphenols, quercetin, resveratrol, *Saccharomyces cerevisiae*.

Abstract

Polyphenols form a group of important natural bioactive compounds with numerous ascribed health-beneficial attributes (*e.g.* antioxidant, anti-inflammatory, anti-microbial and tumor-suppressing properties). Some polyphenols can also be used as natural dyes or plastic precursors. Notwithstanding their relevance, production of most of these compounds still relies on extraction from plant material, which for most of it is a costly and an inefficient procedure. The use of microbial cell factories for this purpose is an emerging alternative that could allow a more efficient and sustainable production. The most recent advances in molecular biology and genetic engineering, combined with the ever-growing understanding of microbial physiology have led to multiple success stories. Production of multiple polyphenolic compounds or their direct precursors has been achieved not only in the common production hosts, such as *Escherichia coli* and *Saccharomyces cerevisiae*, but also in *Corynebacterium glutamicum* and *Lactococcus lactis*. However, boosting production of native compounds or introduction of heterologous biosynthetic pathways also brings certain challenges, such as the need to express, balance and maintain efficient precursor supply. This review will discuss the most recent advances in the field of metabolic engineering of microorganisms for polyphenol biosynthesis and its future perspectives, as well as outlines their potential health benefits and current production methods.

Introduction

There exist over 200,000 different secondary metabolites in plants [1, 2]. Of those, polyphenols are among the most widespread and ubiquitous classes. It has been estimated that in some cases up to 20% of the fixed carbon goes into the phenylpropanoid pathway that leads to the production of the majority of naturally-occurring phenolic compounds [3]. Although polyphenols are classified as secondary metabolites, i.e. molecules that in plants play little or no role in primary metabolism and therefore are not essential for cell's survival under normal conditions, these compounds may accumulate in considerably high amounts [4]. Polyphenols perform many diverse functions in plants, including anti-microbial and anti-fungal protection, insect feeding deterrence, providing coloration to leaves, flowers, and fruits, attraction of pollinators, chelation of toxic heavy metals, protection from UV radiation-induced damage, and free radical scavenging [5–8].

In plants, the aromatic amino acids $_{L}$-phenylalanine and $_{L}$-tyrosine are the two biosynthetic precursors of phenylpropanoid compounds (**Fig. (1)**), [9]). This group consists of compounds with the C_6-C_3 backbone, such as cinnamic acid derivatives, coumarins, and lignans [10]. This backbone can be further extended with up to three two-carbon units derived from malonyl-CoA generating various polyphenols, such as curcuminoids, flavonoids, stilbenes, and styrylpyrones. Of those, the flavonoids (C_6-C_3-C_6 backbone) are the largest group. The vast chemical diversity of flavonoids arises from differences in the backbone structure, as well as from a variety of modifications to the backbone. Possible modifications are acetylation, aryl-migrations, glycosylations, hydroxylations, methylations, polymerizations, and prenylations. Based on these variations, the flavonoids can be further subdivided into aurones, flavanones, flavones, isoflavones, flavonols, flavan-3-ols, anthocyanidins, and tannins (**Fig. (1)**), [10]). A good example of the diversity of the decorations is provided by the anthocyanins, most of which are anthocyanidins glycosylated at the position 3 of the C-ring. Anthocyanins may have additional functional groups such as glycosyl groups (*e.g.* 5-glycosylation and 3'-glycosylation), methyl groups (*e.g.* 3′-methylated petunidin and 7, 3'-methylated rosinidin), hydroxyl groups (which distinguish pelargonidin, cyanidin, and delphinidin derivatives), and acyl groups (on glycosyl moieties, could be both aromatic and/or aliphatic). These decorations can profoundly affect chemical properties such as color, hydrophobicity, and stability, as well as have a strong impact on the compounds bioavailability and bioactivity [11–14].

Polyphenols exhibit an immense natural chemical diversity and appear to have a number of different molecular targets, participating in several signaling pathways, and exhibiting pleiotropic activities both in plants and inside the human body when taken up as a part of diet [13, 15–20]. In addition, their occurrence in plants as complex mixtures makes it possible to take advantage of additive or synergistic activities of such combinations [21–23]. As a result of their diversity in structure and the ethnic knowledge of the use of particular plants in traditional medicine [24, 25], polyphenols have been the subject of intense research with respect to their health benefits [26–28]. Along with their use as pharmaceuticals, polyphenols have found many other potential applications such as natural pigments and food colorants, preservatives, monomers for bioplastics and composites, etc. [29–32].

In recent years the global market for polyphenols has seen continuous growth, with the main booster being the accumulated evidence of polyphenols' health-promoting traits leading to increased sales of polyphenols-containing food supplements, cosmetics, and other type of pharma- and nutraceutical products [33, 34]. In order to keep up with the increasing demand, there is a need for innovative solutions to the large-scale production of such compounds that can replace the less economic and eco-friendly traditional production methods, such as direct purification from plant raw materials or chemical synthesis. In this scenario, bio-based production of chemicals through metabolic engineering of microorganisms has emerged as a viable, affordable, and sustainable alternative. The advent of functional "Omics", genome-scale modeling, and high-throughput screening technologies, combined with the ever-growing genome engineering, editing, and (heterologous) gene expression toolbox, has brought metabolic engineering to a more systematic and global level, thus significantly reducing the costs associated with the complete development of a novel bioprocess. The variety and complexity of chemicals that can now be produced using microbial cell factories has remarkably increased allowing now even the production of complex polyphenols with multiple biosynthetic pathways steps [35, 36].

This review will first focus on health benefits of different polyphenol groups and their applications. The current extraction and production approaches will then be discussed. Lastly, the ongoing research towards development of alternative production methods with a special emphasis on harnessing the potential of microbes as cell factories for the biosynthesis of polyphenols will be covered.

Polyphenols, their health-beneficial effects, and potential applications

Although primarily known as anti-oxidants, polyphenols possess multiple other health-beneficial properties. Most notably, there are several studies that recommend daily intake of high levels of polyphenols as a mean of reducing risks of cardiovascular disorders and type II diabetes [37–39]. Furthermore, these compounds were demonstrated to have an effect on certain types of cancer, neurodegenerative diseases, allergies, inflammation, and also to alleviate menopausal symptoms by estrogen mimicking [16, 40–44]. Additionally, polyphenols were documented to have anti-inflammatory, anti-aging, anti-angiogenic properties, as well as anti-viral and antimicrobial activities against human pathogens [45–50]. An overview of the specific health-promoting properties of the major groups of polyphenols is given in Table 1.

Bioactivities of polyphenols extend beyond the field of pharmaceutics, and these compounds are also known for their many applications in food and cosmetics industries as natural substituents for synthetic ingredients. The concern over the safety of synthetic colorants raised by consumer groups [51, 52], combined with the health benefits provided by the many naturally-occurring pigments, has triggered the current marketing trend for replacement of artificial food dyes with natural colors [53]. In this respect, the bright and diverse colors of anthocyanins (hue range from red to bluish-red to purple), together with their health-beneficial properties, relative stability, and high solubility in water, make these flavonoids excellent candidates for food applications (Table 1) [12, 54–56]. As multiple anthocyanins are approved by the European Food Safety Authority [57], a great demand for their use as potential substitutes for the banned synthetic dyes is expected [58–60]. Similarly to colorants, the search for natural solutions to replace currently-used preservatives is also ongoing. Processed foods with minimal synthetic additives and thermal treatment are becoming an increasing trend. Thus, innovative solutions to extend product shelf-life, such as the use of natural preservatives, are required [61, 62]. The antimicrobial activity of flavonoids is a general attribute of this group of compounds, with some specific flavonoids also possessing anti-viral and anti-fungal properties [47, 63]. Moreover, being strong antioxidants [8, 64] flavonoids could potentially protect food from some undesirable chemical changes. Altogether, these attributes make flavonoids a very promising new group of natural preservatives suitable for use in food industry. Cosmetics is another area of interest for polyphenol applications. Multiple plant species that are enriched in polyphenols, such as cocoa, grape, olive, and tea, are used in cosmetic

products [65–67]. The potential of the application of coffee polyphenolic extract and of caffeic acid alone as components for skin care formulations has also been studied [68, 69]. Apart from protecting skin from UV radiation and reactive oxygen species, polyphenols have several other beneficial effects, including antiaging (via inhibition of lipoxygenase, cyclooxygenase, and skin re-modeling enzymes elastase and collagenase), depigmenting (via inhibition of tyrosinase), inhibition of inflammatory responses, and anti-microbial activity [66, 70, 71]. These data provide a good foundation for further research directed towards the application of purified polyphenolic compounds (single or as mixtures) in skincare products.

Current approaches/methods for the production of polyphenols

At the moment all purified polyphenolic compounds available on the market are either obtained through extraction from plant sources (*e.g.* fruits, leaves, or roots), or by total or partial chemical synthesis. So far, resveratrol remains the only exception to these traditional production methods that is also produced by microbial synthesis using *Saccharomyces cerevisiae* (Table 2). The extraction procedures impose numerous limitations that hinder the exploitation of polyphenols for pharmaceutical and biotechnological applications at their full potential [72, 73]. Factors such as low natural abundance inside plant tissues, environmental and geographical conditions, seasonal variation, and the need for complex downstream processing could have a significant impact on the extraction yields of polyphenols. Consequently, the extraction procedures from plant sources are generally labor-intense, costly, and using plant sources may consume large amounts of resources, such as water and land. Furthermore, the resulting preparations tend to contain impurities. One of the best known examples is the laxative emodin that is often co-purified with and contaminates resveratrol extracts from Japanese knotweed (*Polygonum cuspidatum* Siebold & Zucc.) [74]. Furthermore, despite all the progress made in the field of metabolic engineering of plants and plant cell factories for increased production of native secondary metabolites, their application as production hosts for polyphenols is still limited [75–79]. On the other hand, chemical synthesis of polyphenols has limited options for large-scale production due to the high structural complexity of these molecules. Both d*e novo* synthesis and synthesis from purified precursors involves the use of hazardous and toxic chemical solvents, as well as extreme reaction conditions, thus limiting their application to specialized small-scale production [80]. Molecular chirality imposes additional challenges to chemical synthesis, as this process is not stereo-specific and yields a mixture of *R*-

and *S*-stereoisomers, whereas only 2*S*-stereoisomers of polyphenols were shown to be bioactive [81]. Consequently, an extra purification step is required for separating the isomers further reducing the final yield. Hence, more modern and environmentally-minded approaches are required in order to meet the growing demands for these phytochemicals.

Microbial production of plant polyphenols: past achievements and ongoing research

The inefficiency of traditional production methods is a major obstacle to broadening the range of applications for added-value polyphenols, and consequently successful commercialization would require implementation of large-scale, cost-effective, and sustainable production processes. In light of that, construction of designer microorganisms serving as biological platforms for the production of phenolic compounds is becoming a promising alternative. Industrial workhorses, such as *Bacillus subtilis, Escherichia coli* or *Saccharomyces cerevisiae*, have been used for decades in numerous bioprocesses, including biological production of compounds with applications in pharmaceutical, food, and chemical industries. Microbial production of fine chemicals presents multiple advantages that have been reviewed previously [82–84]. Briefly, as compared to plants and plant cell cultures, the microorganisms that are used for production are usually fast-growing and easy to cultivate, which greatly reduces processes costs and production times. They are also able to grow in diverse media, including industrial and agricultural waste, which makes the bioproduction more sustainable. Moreover, microbial fermentations are readily scalable from laboratory through demonstration to commercial production scales. Also, the ease of genetic manipulation with these organisms and the availability of molecular tools (*e.g.* for expression of heterologous polyphenol pathway genes, for manipulation of homologous polyphenol pathway genes, or for genome editing) facilitates the construction of microbial cell factories tailored for production of nearly any natural (and even non-natural) metabolite imaginable [83]. Furthermore, the use of microbial hosts for the production of polyphenols simplifies the product purification procedure, as their secondary metabolism is generally much simpler and competing pathways can be eliminated or deactivated. Lastly, as opposed to the traditional methods for obtaining natural products, microbial-based production can be a lot more environmentally-friendly, as the use of organic solvents or other harsh chemicals for product purification can be reduced [85, 86]. Production of fine chemicals

using microorganisms also requires considerably less natural resources, such as extensive land and water usage, as well as fertilizers and pesticides, needed to obtain and process large amounts of raw plant material [87, 88].

Over the past years, multiple studies demonstrated the potential of microbial cell factories for the production of diverse classes of plant natural products (reviewed in [73, 83]). Among the polyphenols, various flavonoids [35, 83], stilbenes [89], raspberry ketone, a raspberry flavor molecule [90], caffeic acid, a lignin precursor [91, 92], and curcuminoids [93, 94] have been heterologously produced in microorganisms. The most interesting examples of polyphenol production using microbial cell factories are summarized in Table 3. There is also an example of production titers, productivity, and yield that meet the targets of the large-scale commercialization being achieved. The Swiss company Evolva is manufacturing resveratrol using yeast (*S. cerevisiae*) as the production platform [95]. Up to now their EveResveratrol™ remains the only marketed phenolic compound produced *de novo* by fermentation in metabolically-engineered microorganisms (Table 2). It is also noteworthy that Evolva has either already established, or is about to initiate microbial production of several other phytochemicals, including the flavor and fragrance ingredient vanillin, the spice saffron, and the natural stevia sweeteners (http://www.evolva.com/products/). This example clearly demonstrates the feasibility of commercial bio-based production of plant-borne compounds in microbial cell factories.

From early days, the health benefits ascribed to polyphenols have prompted a significant amount of research work towards the elucidation of their biosynthetic pathways, the genes involved, and their regulation. The information gathered over the past years has led to multiple cases of successful genetic and/or metabolic engineering of whole plants or of plant cell cultures for improved biosynthesis of native and non-native polyphenolic compounds (for selected reviews see [33, 75, 77, 96–99]). This section, however, will only focus on the latest developments in the field of polyphenol production by microorganisms, since these are probably the better candidates for a large-scale and sustainable production.

The first steps of the phenylpropanoid biosynthesis pathway lead to the production of *p*-coumaric acid via deamination of the aromatic amino acids $_{L}$-phenylalanine or $_{L}$-tyrosine (**Fig. (1)**). Production of *p*-coumaric acid from $_{L}$-phenylalanine is a two-step process where the amino acid is first converted into *trans*-cinnamic acid by a phenylalanine ammonia-lyase (PAL), which is further hydroxylated by cinnamate 4-hydroxylase (C4H), a

cytochrome P450 enzyme. The successful production of *p*-coumaric acid from L-phenylalanine was first demonstrated in *S. cerevisiae* by co-expression of PAL- and C4H-coding genes [100]. However, expression of this pathway in bacteria presents a challenge due to involvement of the P450 enzyme. These proteins are usually membrane-bound, thus functional expression in prokaryotes is difficult due to lack of the endoplasmic reticulum. Also, cytochromes P450 rely on P450 reductase enzymes (CPR) for cofactor regeneration, which are not present in bacteria and therefore need to be co-introduced into production strains [36]. In contrast, conversion of L-tyrosine into *p*-coumaric acid occurs in a single step catalyzed by a tyrosine ammonia-lyase (TAL), circumventing the need for C4H activity. A recent study describes several novel highly-specific TAL enzymes that are functional and produce high levels of *p*-coumaric acid in *E. coli*, *Lactococcus lactis*, as well as in *S. cerevisiae* [101]. Alternatively, use of a promiscuous PAL that can also take up L-tyrosine as a substrate for the production of the flavanone naringenin has been reported in *E. coli* [102, 103].

Flavonoids are by far the most explored group of polyphenols in terms of heterologous production in microorganisms [104]. *De novo* biosynthesis of complex flavonoids would require efficient production of the flavonoid core molecules, flavanones. These compounds are synthesized by CoA-esterification of cinnamates, such as *p*-coumaric acid and cinnamic acid, by 4-coumaroyl-CoA ligase (4CL), followed by condensation with three malonyl-CoA molecules catalyzed by chalcone synthase (CHS) and subsequent ring closure by chalcone isomerase (CHI). Further chemical modifications of flavanones, such as hydroxylations, methylations, methoxylations, acylations, and glycosylations, give rise to the vast diversity of flavonoid compounds (**Fig. (1)**). Flavanones such as naringenin and pinocembrin were successfully produced in *E. coli* and *S. cerevisiae* by co-expression of different combinations of PAL/TAL, 4CL, CHS, and CHI enzymes (for an overview see [35, 105–107]). Biosynthesis of a more complex flavanone, eriodictyol, has also been engineered in *E. coli* by additional expression of a flavonoid 3'-hydroxylase (F3'H) enzyme [108]. Furthermore, by combining 4CL, CHS, CHI, and chalcone reductase (CHR), liquiritigenin, 7-hydroxyflavanone, and butin were produced from, respectively, *p*-coumaric acid, cinnamic acid, and caffeic acid as precursors in both *E. coli* and *S. cerevisiae* [109]. To further broaden the spectrum of microbially-produced flavonoids, the biosynthesis of flavones [110, 111] and isoflavones [112] from precursors was achieved through the additional introduction of flavone synthase (FNS) or isoflavone synthase (IFS) genes, respectively. In another study, isoflavone genistein was produced directly from L-phenylalanine in yeast [107] and from L-tyrosine in *E. coli* [113]. Co-expression of the above-mentioned flavanone biosynthetic genes with flavanone

3-hydroxylase (F3H)- and flavonol synthase (FLS)-coding genes yielded the flavonols, kaempferol from L-tyrosine and galangin from L-phenylalanine [114]. Similarly, production of the flavonol fisetin from L-tyrosine has been recently established in *E. coli* by combining the liquiritigenin biosynthesis genes with the genes coding for F3H, FLS, and F3'H [115]. By combining F3H, dihydroflavonol reductase (DFR) and leucocyanidin reductase (LAR), the production of flavan-3-ols (+)-catechin and (+)-afzelechin was achieved from caffeic acid and *p*-coumaric acid, respectively [116]. Lastly, flavanones have also been converted to anthocyanins in a four-step pathway involving F3H, DFR, anthocyanidin synthase (ANS), and anthocyanidin 3-*O*-glucosyltransferase (3GT) [117, 118].

As mentioned above, flavonoids can be further modified by various decorating enzymes. Such modifications not only alter chemical properties and improve stability, but sometimes also grants the compounds novel biological activities [119, 120]. Thus, modified flavonoids might present additional commercial interest. The most common modification of plant flavonoids is glycosylation, often occurring at least at one position [120]. Multiple studies addressed the issues of glucosylation of flavonols [121, 122], flavones [123, 124], flavanones [125], and isoflavones [122, 126, 127]. Addition of other sugar moieties has been also successfully attempted, including rhamnosylation [121, 128, 129], xylosylation [130, 131], and galactosylation [132]. Lastly, biosynthesis of quercetin 3-*O*-*N*-acetylglucosamine has been reported as well [133].

Methylation is another common modification, and there have been several studies aiming at microbial biosynthesis of methylated flavonoids. One such example is the work of Kim *et al*, where *E. coli* strains for the production of ponciretin (4'-*O*-methylnaringenin) and sakuranetin (7-*O*-methylnaringenin) were constructed [134]. Other examples refer to the construction of strains producing the medically-important flavanonol 7-*O*-methyl aromadendrin from *p*-coumaric acid [135] and the flavonol genkwanin (7-*O*-methyl apigenin) from L-tyrosine.

Compared to flavonoids, microbial production of stilbenes is somewhat less of a hot topic. Nevertheless, numerous health benefits attributed to this group of polyphenols did stimulate research efforts to produce them heterologously in microorganisms for various applications in pharmaceutical and food industries. Similarly to flavonoids, stilbenes are produced via decarboxylative condensation of three malonyl-CoA molecules with the CoA-activated hydroxycinnamates through the action of stilbene synthase (STS) (**Fig. (1)**). Original attempts to establish microbial production of stilbene resveratrol were mainly done by the co-expression of the *4cl* and the *sts* genes , and have been accomplished in both *S. cerevisiae* and *E. coli* [136–140]. A more systematic approach comprising the use of two

different production strains, two promoter systems, screening of a *sts* gene library, and fine-tuning of gene expression levels further improved the production of resveratrol from *p*-coumaric in *E. coli* [141]. The engineered strain *E. coli* BW27784 expressing the *4cl gene*from *Arabidopsis thaliana* and the *sts gene* from *Vitis vinifera* organized in a bi-cistronic operon on a high-copy number plasmid, accumulated the impressive amount of 2.4 g/L resveratrol after the addition of a fatty acid biosynthesis inhibitor to improve precursor availability. Research efforts to bypass the use of the expensive precursor *p*-coumaric acid by supplying external $_{L}$-phenylalanine or $_{L}$-tyrosine resulted in consistently low titers of resveratrol [104, 107, 142, 143]. However, extensive strain optimization through i) increase of the availability of $_{L}$-phenylalanine and malonyl-CoA, ii) integration of the resveratrol biosynthetic pathway in the genome and iii) introduction of a resveratrol exporter resulted in a *S. cerevisiae* strain capable of producing 4 g/L of resveratrol from glucose in a fed-batch fermentation [95]. More recently, resveratrol was also produced through *de novo* biosynthesis from both glucose and ethanol via the $_{L}$-tyrosine intermediate at approximately 0.5g/L [144]. Biosynthesis of pinosylvin from $_{L}$-phenylalanine has been also reported [145, 146]. Several other studies focused on the production of methylated resveratrol derivatives, such as the mono-methylated pinostilbene and the di-methylated pterostilbene, which are equally or sometimes even considerably more bioactive than resveratrol [147]. Production of both pinostilbene and pterostilbene from *p*-coumaric acid was achieved by additional expression of stilbene *O*-methyltransferases (OMTs) genes from various sources in both *S. cerevisiae* and *E. coli* [148, 149]. Furthermore, another study has reported production of the unnatural stilbene methyl ethers by the expression of *Oryza sativa* OsOMT1 in *E. coli* [145].

The ever-increasing knowledge of specific pathways and the discovery of novel enzymes have contributed to the microbial production of difficult-to-synthesize polyphenols, such as certain phenolic acids or coumarins, whose biosynthesis involves production of cytochrome P450 enzymes or *O*-methyl-transferases. Identification of several bacteria-compatible hydroxylases that can replace *p*-coumarate 3-hydroxylase (C3H, a P450 enzyme) has made it possible to engineer artificial pathways for the biosynthesis of caffeic and ferulic acids from $_{L}$-tyrosine or *p*-coumaric acid [91, 142, 150–152]. Maximal concentrations of 767 mg/L of caffeic acid and 196 mg/L ferulic acid were produced *de novo* by $_{L}$-tyrosine overproducer strains of *E. coli* expressing, respectively, TAL and 4-hydroxyphenylacetate 3-hydroxylase [150] or TAL, C3H, and a caffeic acid methyltransferase (COM) [152]. Production of plant-specific coumarins in bacteria has been also described [153]. At the first stage, *E. coli* strains were engineered to convert the phenylpropanoid acid precursors, *p*-coumaric acid and ferulic acid, into the simple

coumarins, umbelliferone (4.3 mg/L) and scopoletin (27.8 mg/L), respectively. Furthermore, these two coumarins were later-on produced *de novo* without the addition of any precursor after assembling the complete artificial biosynthetic pathway in *E. coli*. This pioneering study set the foundation for microbial production of more diverse coumarin molecules. Coumarins are components of various polymers [154] and were also demonstrated to have analgesic and anti-inflammatory properties [155], thus their production in microbial cell factories could also be of a commercial interest.

Although the use of *S. cerevisiae* and *E. coli* presents multiple advantages, other microorganisms might be more suitable for production of polyphenolic compounds, for example due to higher end-product tolerance or a broader range of growth substrates. One of the first attempts of using a non-conventional host for the flavonoid production was done in *Streptomyces venezuelae*, where the flavanones naringenin and pinocembrin, as well as the stilbenes resveratrol and pinosylvin were produced from, *p*-coumaric acid and cinnamic acid, respectively [156]. The same organism was later on engineered for the biosynthesis of kaempferol and galangin by the co-expression of the *f3h* gene from *Citrus siensis* and the *fls* gene from *Citrus unshius* and feeding with naringenin or pinocembrin, respectively [157]. A more recent study has demonstrated the feasibility of polyphenol production in *Corynebacterium glutamicum*, a soil bacterium that is used for amino acid production on industrial scale. Kallscheuer *et al*, were able to engineer this bacterium to produce resveratrol and naringenin directly from glucose with yields comparable to those observed in *E. coli* [158]. Moreover, *C. glutamicum* was further engineered for the production of resveratrol from 4-hydroxybenzoic acid (HBA) by reversal of a β-oxidative phenylpropanoid degradation pathway [159]. This allows having polyphenol production independent from the aromatic amino acid biosynthesis. The most "exotic" case was a study where the edible macrofungus *Tremella fuciformis* Berk. (silver ear or white jelly mushroom, division Basidiomycota) was genetically modified for the biosynthesis of resveratrol from *p*-coumaric acid with yields of 0.8-0.9 μg/g of dry weight after 7 days of cultivation [160].

Strategies for improving production of polyphenolic compounds in microorganisms

Metabolic engineering is the introduction of targeted adjustments to cellular metabolic processes aiming at improving production of a certain substance. This is often achieved with a set of genetic manipulations that leads to alterations within various regulatory, enzymatic, and transport functions of the cell [161, 162]. Metabolic engineering is particularly important for optimization of heterologous pathways, as introduction of such pathways

often leads to flux imbalance, not only within the pathway, but also often within the global cellular metabolism. This occurs because the host generally lacks the regulatory machinery required for efficient and balanced operation of the pathway, and also to prevent over- or under-production of enzymes, leading to metabolic burden on the host at the cost of productivity of the compound of interest, as well as accumulation of potentially toxic intermediates [163].

One of the key targets for metabolic pathway engineering is the improvement of precursor supply. The most notable strategies for that are summarized in Table 4. Numerous studies dealing with polyphenol production have concluded that increasing the intracellular pool of malonyl-CoA is the key requirement for enhancing the flavonoid and stilbene production [112, 164, 165]. The most common strategies for that are a) the overexpression of an acetyl-CoA carboxylase (ACC)-coding gene that converts acetyl-CoA into malonyl-CoA and b) the inhibition of fatty acid biosynthesis by addition of the antibiotic cerulenin [35, 146, 166, 167]. Further improvement could be achieved through the fine-tuning of the acetate assimilation via the overexpression of acetyl-CoA synthetase gene and the deletion of the acetate-utilizing pathways, which overall resulted in a 16.3-fold increase of intracellular malonyl-CoA concentration [108, 166]. Other alternatives include the introduction of a malonate catabolic pathway [168, 169], the overexpression of the 3-ketoacyl-ACP synthase genes *fabH* and *fabF* [166, 170], and the conditional down-regulation of fatty acid biosynthesis with CRISPR interference (CRISPRi) [171]. Several other studies in *E. coli* have taken a more global approach, combining computational predictions and experimental validations [165, 172, 173]. The utilized genome-scale models predicted a set of genetic interventions, mainly aiming to up-regulate some of the glycolytic reactions and down-regulate the tricarboxylic acid (TCA) cycle that cooperatively drive the carbon flux towards malonyl-CoA, while at the same time preventing the formation of byproducts. These interventions were experimentally validated using a lab-scale fermenter, and the introduced genetic modifications resulted in a significantly improved production of naringenin (474 mg/L, [172]) and resveratrol (1600 mg/L, [173]).

Other substrates/co-factors critical for the biosynthesis of flavonoids are the UDP-glucose and NADPH [117, 169, 174]. The former one is the donor of the glucosyl group in the anthocyanin biosynthesis as well as in other flavonoid biosynthetic routes, whereas the latter one is required for the biosynthesis of leucoanthocyanidins, 5-deoxyflavanones, and (+)-catechins (**Fig. (1)**). Engineering of UDP-glucose levels through a combination of overexpressing genes of the UTP biosynthetic pathway, supplementation with orotic acid, and deletion of several endogenous UDP-glucose-utilizing genes resulted in the significantly improved anthocyanin production from

342 flavan-3-ols precursors in an *E. coli* strain expressing ANS- and 3GT-coding genes [169]. Recently, a follow-up
343 study has reported that overexpression of the *ycjU* gene, which catalyzes the conversion of β anomer of glucose-6-
344 phosphate into glucose-1-phosphate, further increases the UDP-glucose pools, and consequently anthocyanin
345 production [118]. With regard to improving the intracellular NADPH availability, a stoichiometry-based model was
346 deployed to identify a set of potential gene knock-out combinations in *E. coli*. Upon validation of the candidates, the
347 combined inactivation of phosphoglucose isomerase, phosphoenolpyruvate carboxylase, and phospholipase
348 activities resulted in a 4-fold increase of leucoanthocyanidin production and a 2-fold increase of (+)-catechin
349 production, as compared to the wild-type background [174].

350 Another challenge to the current metabolic engineering strategies is the use of media supplemented with expensive
351 precursors (*e.g. p*-coumaric acid or naringenin) and, a two step-fermentation process (biomass/protein production
352 and polyphenol synthesis) that becomes a disadvantage when the process scale-up is considered. Partly, this issue
353 comes from the fact that biosynthetic pathways for complex polyphenols such as flavonols and anthocyanins consist
354 of six or more genes. Overexpression of such high number of genes would first of all cause a large metabolic burden
355 to the cell. This problem has already been partly addressed by using a co-culture strategy [175]. With this approach,
356 Jones *et al.* have split the (+)-afzelechin biosynthetic pathway between the two co-incubated strains: the first one
357 was expressing the malonyl-CoA-dependent part (from *p*-coumaric acid to naringenin), whereas the second strain
358 was expressing the NADPH-dependent downstream part (from naringenin to (+)-afzelechin). Another advantage of
359 the co-cultivation system is that the two strains could be independently engineered for enhanced precursor supply
360 (*e.g.* malonyl-CoA or NADPH) without significantly impacting the cellular metabolism. One of the major
361 parameters that need to be considered and optimized in such experiments is strain compatibility. The selected strains
362 must have similar growth kinetics to avoid out competition of one, which can lead to an imbalance in the pathway.
363 The authors addressed this issue by introducing the plasmids containing the biosynthetic genes into multiple
364 background strains and selecting the most fitting combination. Equal growth could also potentially be ensured by
365 introducing two different auxotrophies in the production strains in a way that would make them dependent on one
366 another. Furthermore, production of compounds that are toxic to other members of the consortium must be avoided
367 and efficient transfer of pathway metabolites from one partner to another must be ensured. The second issue is that
368 expression of this many genes could be challenging due to lack of compatible sets of overexpression vectors for
369 many industrially-relevant microorganisms. This, however, is not an issue for *E. coli* where the complete

biosynthetic pathway for the flavonol fisetin from L-tyrosine consisting of seven genes has been established using the DUET vector system, in which genes were expressed in pairs utilizing four different expression vectors [115]. There is also an interesting solution in *S. cerevisiae* that involves the use of a polyprotein system allowing co-transcription of multiple coding sequences from a single promoter. The system has already been used for the production of 2-hydroxynaringenin-*C*-glucoside [176]. Furthermore, in order to allow complete *de novo* biosynthesis, both bacterial and yeast strains have been engineered for the production of flavonoids and stilbenes from inexpensive substrates, such as glucose, by introducing heterologous genes coding for various polyphenol biosynthesis pathways into L-tyrosine- or L-phenylalanine-overproducing strains [168, 177–179].

Microbial production of polyphenols is often challenged by toxicity of the end-product and/or of its biosynthetic intermediates, as well as by the formation of inclusion bodies resulting from protein overproduction. The former issue is related to anti-microbial properties of polyphenols, which could become an issue particularly if the produced molecules accumulate intracellularly at high concentrations. One possible approach to resolve this is to co-express an exporter protein that would extrude the produced polyphenols into the culture medium [95]. Another approach is to use adaptive laboratory evolution (ALE), which consists of continuous cultivation of the producer microorganism while subjecting it to increasing concentrations of the polyphenols. This process generally results in accumulation of mutations that would increase tolerance of the producer strain towards the target compound [180, 181]. Furthermore, cytotoxic effect of the biosynthetic intermediates could be avoided by balancing expression levels of individual genes within the given pathway [168, 182]. The second issue arises from the necessity for some enzymes to form complexes in order to ensure high local substrate concentrations, in particularly if a reaction is unfavorable [82]. However, this could also be of an advantage even if the coupling is unnatural, as it would ensure efficient flux from one step of a pathway to another. There are multiple ways of ensure close proximity of biosynthetic enzymes and their intermediates, including intracellular compartmentalization [82], use of synthetic scaffolds [183], and construction of translational fusions. The latter approach has been used multiple times for engineering of polyphenol production, including construction of the 4CL::STS fusion for enhancing resveratrol production [137], construction of CHS::CHR fusion for increasing liquiritigenin production [115], and use of P450::CPR to allow functional expression of P450 enzymes in bacteria [108, 112, 115].

A recent report has drawn the attention to the interference of native aromatic acid degradation pathways with the production of polyphenols [135]. Detailed analysis of *E. coli* strains producing 7-*O*-methyl aromadendrin showed that the final concentration of this flavanonol did not correspond to the consumption of *p*-coumaric acid, indicating possible degradation of the precursor via an unknown pathway [184]. *S. cerevisiae* has been also reported to have more than one enzyme catalyzing decarboxylation of *trans*-cinnamic acid and *p*-coumaric acid, a reaction which could also potentially reduce production of polyphenols [185]. A similar situation was observed in *C. glutamicum* where a phenylpropanoid degradation gene cluster had to be deleted prior to engineering of this bacterium for stilbene and flavonoid production [158, 186].Therefore, possible presence of such enzymes and pathways needs to be accounted for, in particular prior to exploration of a new production host.

Another interesting recent development is the emergence of combinatorial gene expression techniques that appear to be promising approaches to address the challenge of improving titers and productivity efficiency [163, 187]. However, their successful application is highly dependent on the availability of high-throughput methods for strain screening [188]. Recently, two flavonoid biosensors were constructed consisting of the reporter gene coding for the fluorescent protein placed under control of the flavonoid-responsive transcriptional regulator [189]. The transcriptional regulators FdeR from *Herbaspirillum seropedicae* SmR1 was used to generate a biosensor to detect naringenin, whereas QdoR from *B. subtilis* was used to detect quercetin and kaempferol. The QdoR-based biosensor was highly efficient in detecting kaempferol production *in vivo* at the single cell level while using fluorescence-activated cell sorting (FACS). The developed biosensors could be subsequently used for identification of novel genes involved in polyphenol biosynthetic pathways [189]. Another biosensor has been developed based on the *B. subtilis* transcription factor FapR that is responsive to malonyl-CoA [190]. This sensor could therefore be used for selection of candidates with increased intracellular concentrations of malonyl-CoA. Liu *et al*. [191] have used the same transcription factor in order to develop a negative feedback regulatory circuit. The circuit relies on a malonyl-CoA-based sensor-actuator system that controls expression of the *acc* gene, and in this way alleviates the toxic effects of high intracellular concentration of the enzyme. The circuit was proven to be efficient in regulating the fatty acid biosynthetic pathway and increasing fatty acid titer and productivity [191]. Application of such system for microbial production of polyphenols should allow balancing the engineered pathways and subsequently improve the production efficiency. Other approaches and techniques that have been successfully utilized for fine-tuning gene expression for the needs of metabolic engineering were thoroughly reviewed in [192]. Furthermore, a new screening

technique based on high-performance thin-layer chromatography (HPTLC) has been developed for the discovery of flavonoid-modifying enzymes [193]. The authors claim that this metagenome extract thin-layer chromatography analysis (META) allows rapid detection of glycosyltransferases and other flavonoid-decorating enzymes, as well as that the system is highly sensitive, being able to detect of as little as 4 ng of a modified molecule.

Conclusion

There has been a substantial progress in the field of microbial production of polyphenols. The recent advances in genome editing, combined with novel engineering tools, now allow the expression of multiple genes coding for enzymes forming complex biosynthetic polyphenol pathways not only in model organisms such *E. coli* and *S. cerevisiae*, but also in more novel productions hosts, such as *C. glutamicum*, *L. lactis*, and *Streptomyces venezuelae*. Nonetheless, in many cases the production efficiency using microbial hosts remains inferior as compared to extraction from plants. However, constant advances of synthetic biology tools combined with future metabolic engineering efforts will further facilitate the development of more economically-favorable production processes.

Abbreviations

3GT – anthocyanidin 3-*O*-glycosyltransferase, 4CL – 4-coumaroyl-CoA ligase, AAT – anthocyanin acyltransferase, ACC – acetyl-CoA carboxylase, ALE – adaptive laboratory evolution, AMT – anthocyanin methyltransferase, ANR – anthocyanidin reductase, ANS – anthocyanidin synthase (leucoanthocyanidin dioxygenase), C3H – *p*-coumarate 3-hydroxylase, C4H – cinnamate 4-hydroxylase, CDW – cell dry weight, CHI – chalcone isomerase, CHR – chalcone reductase, CHS – chalcone synthase, CoA – Coenzyme A, COM – caffeic acid methyltransferase, CPR – cytochrome P450 reductase, CRISPRi – clustered regularly-interspaced short palindromic repeats interference, CUS – curcuminoid synthase, CVD – cardiovascular diseases, DFR – dihydroflavonol 4-reductase, F3'H – flavonoid 3'-hydroxylase, F3H – flavanone 3-hydroxylase, FACS – fluorescence-activated cell sorting, FLS – flavonol synthase, FNS – flavone synthase, HBA – 4-hydroxybenzoic acid, HTC – high-throughput screening, IFS – isoflavone synthase, K/O – knock-out, LAR – leucoanthocyanidin reductase, NADPH – nicotinamide adenine dinucleotide phosphate, O/E – overexpression, OMT – 3-*O*-methyltransferase, LDL – low-density lipoprotein, PAL – phenylalanine ammonia-lyase, STS – stilbene synthase, TAL – tyrosine ammonia-lyase, TCA cycle – tricarboxylic acid cycle, UDP-glucose – uridine diphosphate glucose.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgments

AD, PG, ARN, and JF wrote the manuscript. All authors read and approved the final manuscript.

The authors would like to thank the European Union's Seventh Framework Programme (BacHBerry, Project No. FP7-613793, and FP7-PEOPLE-2013-COFUND, Project No. FP7-609405) and the Novo Nordisk Foundation for their financial support. The authors acknowledge Dr. Claudia Santos (iBET - Instituto de Biologia Experimental e Tecnológica, Oeiras, Portugal) for her valuable contribution with the information used for preparing Tables 1 and 2 of the manuscript.

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1024 **Table 1. Health-beneficial properties of the major groups of polyphenols.**

Polyphenol	Applications: health-promoting or biotechnological
Phenolic acids *Examples:* Gallic acid Caffeic acid Ferulic acid Chlorogenic acid	**Cancer**: Chemopreventive activity, as well as protection against side effects of chemotherapy [194, 195]. **CVD and Diabetes:** Prevent oxidation of low-density lipoprotein (LDL)-cholesterol and effective in the treatment of hypercholesterolemia and type 2 diabetes [196–198]. **Neurodegenerative diseases**: Potential as agents for the treatment of Alzheimer's disease [199]. **Others:** Anti-allergic; anti-microbial; antioxidant and immunomodulatory activities. Di- and tri-caffeic/quinic acids have antiretroviral activity [200–204].
Anthocyanins *Examples:* Cyanidin Pelargonidin Delphinidin Rosinidin	**Cancer**: Inhibit initiation and progression stages of tumor development; reduce effect of inflammation on promotion of tumorogenesis; suppress angiogenesis; minimize cancer-induced DNA damage [205–207]. **CVD and Diabetes:** Improve vascular health; protect against cardiovascular diseases; anti-obesity effects through improvement of adipocyte function; may contribute to prevention of the metabolic syndrome; potential anti-diabetic activity [64, 205, 208–210]. **Neurodegenerative diseases**: Protection against brain ageing and decline in cognitive performance in animal models [205, 211, 212]. **Others:** Reduce inflammatory biomarkers; bacteriostatic against some gut pathogenic bacteria; food colorants [205, 208, 213, 214].
Flavonols *Examples:* Quercetin Kaempferol Myricitin Fisetin Morin	**Cancer**: Protective effects against pancreatic, breast, cervical, prostate, uterine, urinary tract cancers, and leukemia [49, 215–217]. **CVD and Diabetes:** Confer cardioprotection and improve the levels of risk factors for cardiovascular disease [218, 219]. **Neurodegenerative diseases**: Neuroprotective activity in experimental focal ischemia and models of neurodegeneration; cognition-enhancing; reduce the risk of Alzheimer's disease [220–222].

Rutin	**Inflammation:** Anti-inflammatory [223]. **Others**: Anticonvulsant, antioxidants, memory enhancement [203, 224, 225].
Flavanols *Examples:* Catechins Epigallocatechin Thearubigins Mesquitol	**Cancer**: Inhibition of tumorigenesis in different organs of animals [205, 226]. **CVD and Diabetes:** Cardioprotective effect by reverting of endothelial dysfunctions; decreasing inflammatory biomarkers, and providing antioxidant and antiplatelet effects. Also have beneficial effects on blood pressure, blood glucose level, and lipid parameters [227–230]. **Neurodegenerative diseases**: Neuroprotective/neuroregenerative effects as modulators of intracellular neuronal signaling and metabolism, cell survival/death genes, and mitochondrial function [212, 231].
Hydrolyzable tannins *Examples:* Grandinin Casuarictin Punicalagin Vescalagin	**Cancer**: Anti-tumor, anti-proliferative and anti-mutagenic effects [232, 233]. **CVD and Diabetes:** Anti-diabetic; anti-atherogenic; anti-thrombotic [234, 235]. **Others**: Anti-inflammatory, anti-bacterial, and anti-mycotic properties. Ellagitannins and gallotannins may also affect the life of foodstuff due to their antioxidant properties and/or antimicrobial activity [236, 237].
Isoflavones *Examples:* Genistein Daidzein Curcumin Glycetin	**Cancer**: Inhibition of cell proliferation [47, 238, 239]. **CVD and Diabetes:** Anti-platelet effects [240, 241]. **Others**: Neuroprotective agents; improve cognitive functions and alleviate menopause symptom in females; anti-thyroid activity [242–244].
Proanthocyanidins (Condensed tannins) *Examples:* Epichatechin trimer Selligueain A Procyanidin B3	**Cancer**: Reduce the incidence and progression of cancer (particularly of prostate cancer) [245, 246]. **CVD and Diabetes:** Reduction of CVD incidence due to their antioxidant activity; inhibition of LDL oxidation; vasodilating properties; anti-platelet activity and protection against ischemia-reperfusion injury [245, 247, 248]. **Others**: Proanthocyanidin-rich extracts inhibit viral adhesion and infectivity of the A

	and B influenza viruses, as well as suppress urinary and *Helicobacter pylori* infections, procyanidin B3 has been described as a hair-growth stimulant, selligueain A is a natural sweetener [245, 249–251].
Lignans *Examples:* Secoisolariciresinol Pinoresinol Podophyllotoxin Steganacin	**Cancer**: Anti-carcinogenic effects on multiple types of cancer [252–254]. **CVD and Diabetes:** Associated with a decreased risk of cardiovascular diseases, hypoglycemic properties [255, 256]. **Others**: Inhibition of *H. pylori* motility and steroid hormone metabolism, anti-viral activities [252, 254, 257].
Stilbenoids *Examples:* Resveratrol Pterostilbene Pinosylvin Piceid	**Cancer**: *in vitro* as well as *in vivo* chemopreventive and chemotherapeutic activities, in all three stages of carcinogenesis (initiation, promotion, and progression) [46, 258]. **CVD and Diabetes:** Improve insulin sensitivity, mimics calorie restriction, lower plasma lipoproteins and cholesterol, prevent cell damage induced by oxidative stress and ischemia [259–262]. **Others**: anti-aging and anti-inflammatory activities, relives endotoxemia-associated adrenocortical insufficiency, confer protection against intestinal barrier dysfunction, modulate gut microbiota by favoring increase in lactic acid bacteria counts [46, 214, 263–266].

1025 CVD – cardiovascular diseases

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1027 **Table 2. Examples of polyphenol-containing products accessible to consumers.**

Type of polyphenol	Production method	Examples of products on the market (supplier)
Phenolic acids (Hydroxycinnamic and hydroxybenzoic acids)	• Extraction from plants	• GCA™-Green Coffee Antioxidant (Applied Food Sciences)
Anthocyanins	• Extraction from plants	• Freeze Dried Polyphenol Fruitbasket (BerryPharma)
	• Extraction from bilberry	• Mirtoselect® and Myrtocyan® (Indena®)
	• Extraction from bilberry	• NutriPhy® Bilberry 100 (Chr. Hansen A/S)
Flavonols (Quercetin/kaempferol/ myricetin)	• Chemical synthesis	• Quercitin complex (Solgar)
	• Extraction from plants	• Bayberry Bark Extract Myricetin (Cactus Botanics)
Flavanols (Catechins)	• Extraction from plants	• Green Tea Catechins, Decaf - *Camellia sinensis*, (Amax)
	• Extraction from plants	• NutraSource, AssuriTEA Green, (Kemin Health)
	• Extraction from plants	• Theaflavin Black Tea Extract (Applied Food Sciences)
Hydrolyzable tannins (Casuarictin)	• Extraction from plants	• PomActiv™ Pomegranate Extract (Cyvex Nutrition)
Isoflavones (Genistein)	• Extraction from soy	• geniVida® (DSM)
Proanthocyanidins (Epichatechin trimer)	• Extraction from plants	• Pine Bark 95% Proanthocyanidins (Cactus Botanics)

	• Extraction from plants	• ENOVITA® - grape seed extract and proanthocyanidin A2 phytosome (Indena)
Lignans (Secoisolariciresinol)	• Extraction from plants • Extraction from plants	• Flaxseed Lignans (Cactus Botanics) • ActiFlax (Marco Hi-Tech)
Stilbenes (Resveratrol)	• Extraction from plants • Chemical synthesis • Microbial production	• Rexatrol® - resveratrol phytosome® (Indena) • ResVida (DSM) • EveResveratrol™ (Evolva)

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1030 **Table 3. Production of polyphenolic compounds in microbial hosts.**

Compound	Precursor	Host	Reference	Highest titer
Phenolic acids				
p-coumaric acid	L-Phenylalanine	*S. cerevisiae*	[100]	~ 7.2 mg/L
	L-Tyrosine	*S. cerevisiae*	[179]	1.93 g/L
	L-Tyrosine	*E. coli*	[101, 152]	1.6 mmol/g CDW
		S. cerevisiae	[101]	133 μmol/g CDW
		L. lactis		43 μmol/g CDW
Caffeic acid	L-Tyrosine	*E. coli*	[151, 152]	150 mg/L
	Glucose	*E. coli*	[91, 150]	767 mg/L
Ferulic acid	L-Tyrosine	*E. coli*	[152]	196 mg/L
Flavanones				
Naringenin	L-Tyrosine	*E. coli*	[102, 103, 164]	57 mg/L
	L-Phenylalanine	*S. cerevisiae*	[107]	8.9 mg/L
	p-Coumaric acid	*E. coli*	[172]	474 mg/L
	Glucose	*E. coli*	[178]	84 mg/L
	Glucose	*C. glutamicum*	[158]	32 mg/L
	Glucose	*S. cerevisiae*	[177]	113 mg/L
	p-Coumaric acid	*St. venezuelae*	[156]	4 mg/L
Pinocembrin	L-Phenylalanine	*E. coli*	[102, 164]	58 mg/L
	Glucose	*E. coli*	[168]	40 mg/L
	Cinnamic acid	*St. venezuelae*	[156]	6 mg/L
Eriodictyol	L-Tyrosine	*E. coli*	[108]	43 mg/L
Liquiritigenin	*p*-Coumaric acid	*E. coli*	[109]	17 mg/L
		S. cerevisiae		14 mg/L

7-hydroxyflavanone	Cinnamic acid	*E. coli*		1.9 mg/L
		S. cerevisiae		0.9 mg/L
Butin	Caffeic acid	*E. coli*		4.2 mg/L
		S. cerevisiae		2.5 mg/L
Sakuranetin	L-Tyrosine	*E. coli*	[134]	40 mg/L
Ponciretin	L-Tyrosine	*E. coli*	[134]	43 mg/L
Flavones				
Chrysin	Cinnamic acid	*S. cerevisiae*	[110]	0.9 mg/L
	L-Phenylalanine	*E. coli*	[114]	9 mg/L
Apigenin	*p*-Coumaric acid	*S. cerevisiae*	[110]	0.4 mg/L
	L-Tyrosine	*E. coli*	[111, 114]	30 mg/L
Genkwanin	L-Tyrosine	*E. coli*	[111]	41 mg/L
Luteolin	Caffeic acid	*S. cerevisiae*	[110]	1.6 mg/L
Isoflavones				
Genistein	Naringenin	*E. coli*	[112]	10 mg/g CDW
	L-Phenylalanine	*S. cerevisiae*	[107]	0.1 mg/L
Daidzein	Liquiritigenin	*E. coli*	[112]	18 mg/g CDW
Flavonols				
Kaempferol	L-Tyrosine	*E. coli*	[114]	15 mg/L
	Naringenin	*St. venezuelae*	[157]	0.2 mg/L
	L-Phenylalanine	*S. cerevisiae*	[107]	1.3 mg/L
Galangin	L-Phenylalanine	*E. coli*	[114]	1.1 mg/L
	Pinocembrin	*St. venezuelae*	[157]	1.0 mg/L
Fisetin	L-Tyrosine	*E. coli*	[115]	0.3 mg/L

Quercetin	*p*-Coumaric acid	*S. cerevisiae*	[107]	0.26 mg/L
7-O-methyl aromadendrin	*p*-Coumaric acid	*E. coli*	[135]	3 mg/L
Flavan-3-ol				
(+)-catechin	Caffeic acid	*E. coli*	[116]	0.09 mg/L
(+)-afzelechin	*p*-Coumaric acid	*E. coli*	[116]	0.04 mg/L
Anthocyanins				
Pelargonidin 3-*O*-glucoside	Naringenin	*E. coli*	[267]	6 μg/L
Cyanidin 3-*O*-glucoside	Eriodictyol	*E. coli*	[267]	6 μg/L
	(+)-catechin	*E. coli*	[118]	350 mg/L
Stilbenes				
Resveratrol	*p*-Coumaric acid	*S. cerevisiae*	[136–138, 140]	391 mg/L
	Glucose	*S. cerevisiae*	[144]	531 mg/L
	Glucose	*S. cerevisiae*	[95]	4 g/L
	p-Coumaric acid	*E. coli*	[139, 141, 173]	1600 mg/L
	Glucose	*C. glutamicum*	[158]	59 mg/L
	p-Coumaric acid	*St. venezuelae*	[156]	0.4 mg/L
	p-Coumaric acid	*T. fuciformis*	[160]	0.8 μg/g CDW
Pinosylvin	L-Phenylalanine	*E. coli*	[145, 146]	91 mg/L
	Glycerol	*E. coli*	[171]	47 mg/L
	L-Phenylalanine	*St. venezuelae*	[156]	0.6 mg/L
Pterostilbene	*p*-Coumaric acid	*E. coli*	[148]	50 mg/L
Pinostilbene	*p*-Coumaric acid	*E. coli*	[149]	34 mg/L

1031 CDW – cell dry weight

1032 **Table 4: Metabolic engineering strategies used for improving precursor supply for polyphenol biosynthesis**

Target	Approach	Host organism	References
Malonyl-CoA pool			
	Addition of cerulenin	*E. coli*	[146, 170, 173, 178]
		C. glutamicum	[158]
	O/E of ACC	*E. coli*	[108, 166, 172]
		S. cerevisiae	[144]
	O/E of acetyl-CoA synthase	*E. coli*	[108, 166]
	K/O of acetate kinase	*E. coli*	[108, 166]
	O/E of *fabF* and *fabE*	*E. coli*	[166, 170]
	Expression of MatB and MatC from *Rhizobium trifolii*	*E. coli*	[168, 169]
	Repression of *fabD*	*E. coli*	[171]
	Up-regulation of glycolysis and down-regulation of the TCA cycle	*E. coli*	[165, 172, 173]
UDP-glucose availability			
	O/E of *pgm*, *galU*, *ndk*	*E. coli*	[169]
	K/O of *galE* and *galT*	*E. coli*	[169]
	O/E of *ycjU*	*E. coli*	[118]
NADPH availability			
	Deletion of *pgi*, *ppc*, and *pldA*	*E. coli*	[174]
Aromatic amino acid availability			

	Modifications of the shikimate pathway	*E. coli*	[168]
		S. cerevisiae	[177, 179]
	Reducing flux through the Ehrlich pathway	*S. cerevisiae*	[177]

1033 O/E – overexpression, K/O – knock-out, TCA cycle – tricarboxylic acid cycle

1034

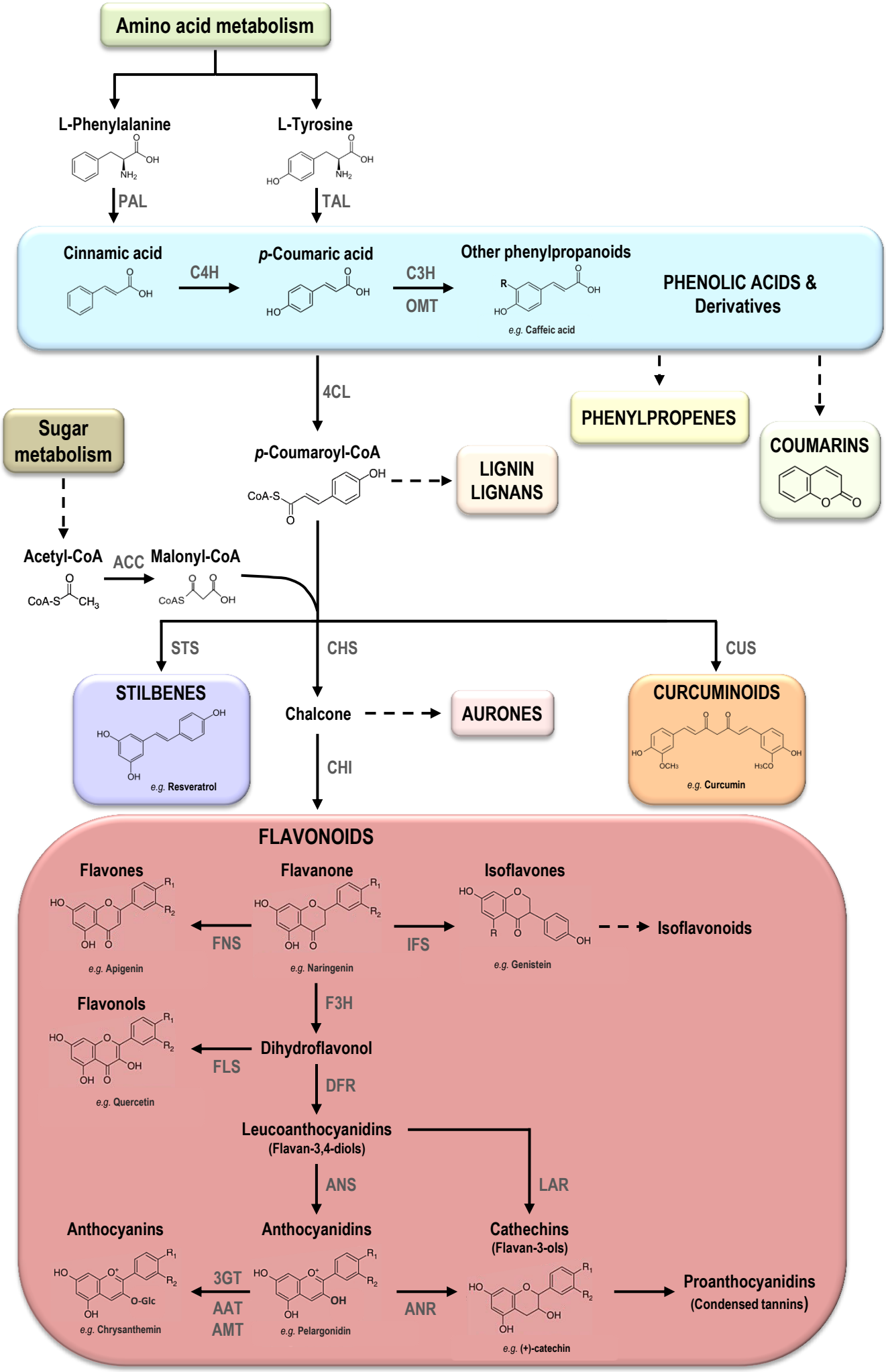
Amino acid metabolism
L-Phenylalanine
L-Tyrosine
PAL
TAL
Cinnamic acid
C4H
p-Coumaric acid
C3H
OMT
Other phenylpropanoids
e.g. Caffeic acid
PHENOLIC ACIDS & Derivatives
4CL
PHENYLPROPENES
COUMARINS
Sugar metabolism
p-Coumaroyl-CoA
LIGNIN LIGNANS
Acetyl-CoA
ACC
Malonyl-CoA
STS
CHS
CUS
STILBENES
e.g. Resveratrol
Chalcone
AURONES
CURCUMINOIDS
e.g. Curcumin
CHI
FLAVONOIDS
Flavones
e.g. Apigenin
FNS
Flavanone
e.g. Naringenin
IFS
Isoflavones
e.g. Genistein
Isoflavonoids
F3H
Flavonols
e.g. Quercetin
FLS
Dihydroflavonol
DFR
Leucoanthocyanidins
(Flavan-3,4-diols)
ANS
LAR
Anthocyanins
e.g. Chrysanthemin
3GT
AAT
AMT
Anthocyanidins
e.g. Pelargonidin
ANR
Cathechins
(Flavan-3-ols)
e.g. (+)-catechin
Proanthocyanidins
(Condensed tannins)

Figure 1. Plant polyphenols and their biosynthetic routes. Names of enzymes: 3GT, anthocyanidin 3-*O*-glycosyltransferase; 4CL, 4-coumaroyl-CoA ligase; AAT, anthocyanin acyltransferase; AMT, anthocyanin methyltransferase; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase (leucoanthocyanidin dioxygenase); C3H, *p*-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; CUS, curcuminoid synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; IFS, isoflavone synthase; LAR, leucoanthocyanidin reductase;OMT, 3-*O*-methyltransferase; PAL, phenylalanine ammonia-lyase; STS, stilbene synthase; TAL, tyrosine ammonia-lyase ([1–3]).

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