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Review

Lignin Valorization: Production of High Value-Added Compounds by Engineered Microorganisms

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Abstract: Lignin is the second most abundant polymer in nature, which is also widely generated during biomass fractionation in lignocellulose biorefineries. At present, most of technical lignin is simply burnt for energy supply although it represents the richest natural source of aromatics, and thus it is a promising feedstock for generation of value-added compounds. Lignin is heterogeneous in composition and recalcitrant to degradation, with this substantially hampering its use. Notably, microbes have evolved particular enzymes and specialized metabolic pathways to degrade this polymer and metabolize its various aromatic components. In recent years, novel pathways have been designed allowing to establish engineered microbial cell factories able to efficiently funnel the lignin degradation products into few metabolic intermediates, representing suitable starting points for the synthesis of a variety of valuable molecules. This review focuses on recent success cases (at the laboratory/pilot scale) based on systems metabolic engineering studies aimed at generating value-added and specialty chemicals, with much emphasis on the production of *cis*,*cis*-muconic acid, a building block of recognized industrial value for the synthesis of plastic materials. The upgrade of this global waste stream promises a sustainable product portfolio, which will become an industrial reality when economic issues related to process scale up will be tackled.

Keywords: lignin valorization; biocatalysis; *cis*,*cis*-muconic acid production; aromatic compounds; value-added compounds; metabolic engineering; platform chemical; microbial cell factory

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1. Introduction

Lignin, the second most abundant polymer on Earth, is a main component of lignocellulose in plants. Lignin is a complex polymer assembled from aromatic building blocks, which represents the most underutilized renewable. About 300 billion tons of lignin are available in the biosphere, thus providing a huge resource for sustainable processes [1]. Lignin is also largely produced by various production systems: in biorefinery plants, when one liter of cellulosic ethanol is produced, about one kilogram of lignin is also generated. Lignin is a main by-product of the paper and pulp industry. For example, Kraft pulping generates about 130 million tons of lignin per year [2,3]. At present, most lignin is burnt for energy supply or discarded into the environment: partial valorization of lignin to target chemicals represents a main environmental benefit [4].

Lignin is mainly composed of three phenylpropanoid units: the monolignols coniferyl alcohol (G), sinapyl alcohol (S) and *p*-coumaryl alcohol (H). About 50–80% of all interunit bonds are β-O-4 ether bonds and α-O-4, β-5, β-β and 5–5 linkages and biphenyl and diaryl ether structures are also present (Figure 1) [5]. Lignin content and composition strongly depend on the plant source [6]. Because of its heterogeneous character, lignin depolymerization is a difficult task that generates a wide range of aromatic compounds.

Several excellent reviews reported about catalytic and biological methods for lignin depolymerization [5,7–10]. As a general rule, biological systems seem better suited to handling heterogeneous mixtures than physico-chemical methods: the lignin-degrading

microbes utilize funneling pathways to channel multiple monolignols into key intermediates [11], thus allowing the production of a platform of chemicals. These processes guarantee sustainable green alternatives to fossil routes and also enhance the efficiency of the existing second generation biorefineries. For a comparison of chemical and biological methods for lignin depolymerization, see Table 1.

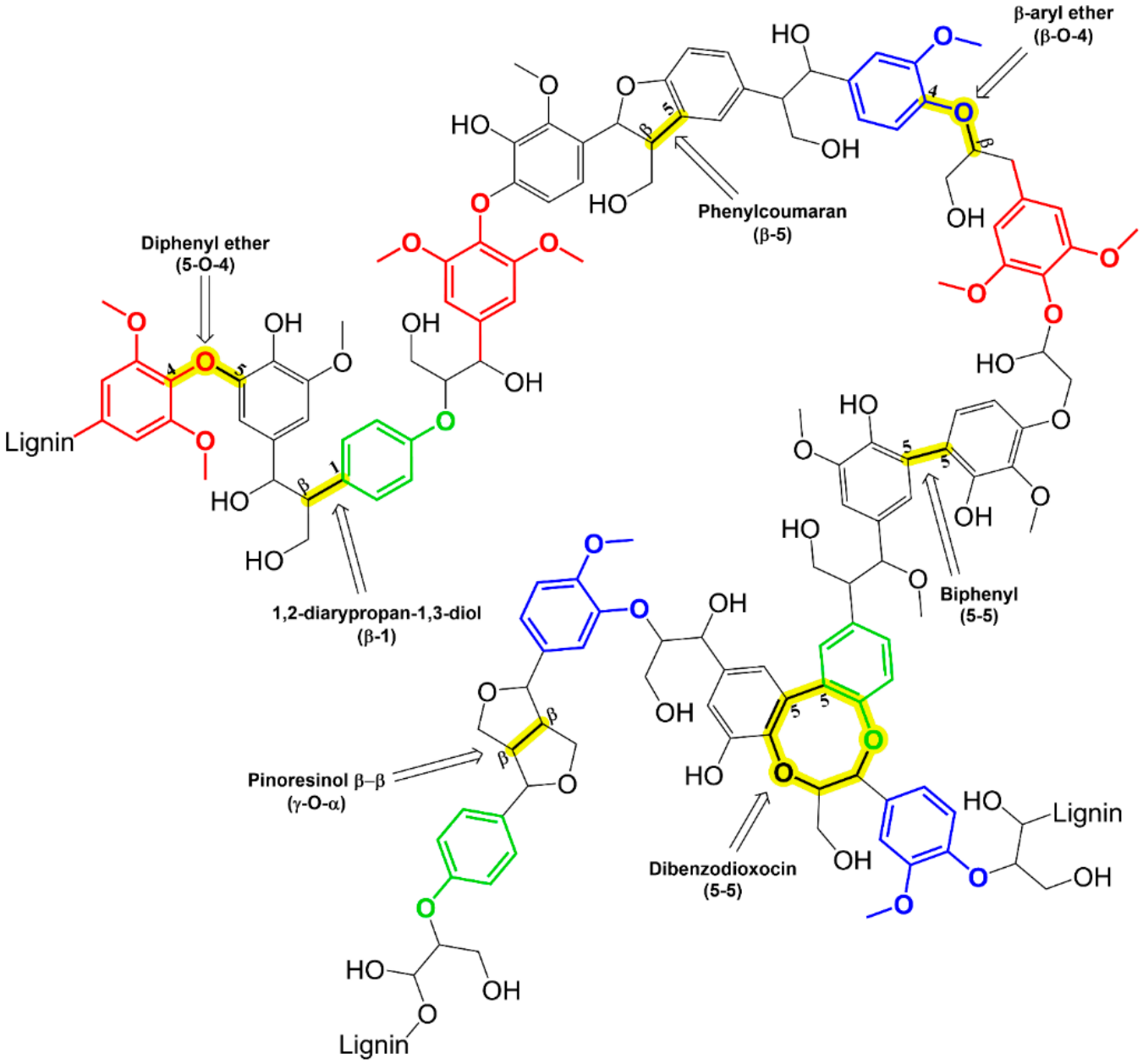

Figure 1. Schematic representation of the lignin structure. The figure shows the three main moieties: *p*-hydrophenyl (H) (green), guaiacyl (G) (blue) and syringyl (S) (red). The most frequent bonds are highlighted in yellow.

White-rot and brown-rot fungi have long been considered the only lignin-metabolizing organisms [12,13]. However, a number of investigations reported on bacteria as lignin-degrading organisms [13], especially the ones belonging to phyla *Proteobacteria*, *Actinobacteria*, and *Firmicutes*, as well as members of the archaeal phylum *Bathyarchaeota* [13–16]. Biological lignin depolymerization is mainly due to extracellular oxidases, such as lignin peroxidase, manganese peroxidase, versatile peroxidase, dye-decolorizing peroxidase, and

laccase [17–20]. Once simple aromatics are released from lignin breakdown, additional microbes are allowed to participate. Notably, aromatics from G-type and H-type lignin are funneled only into protocatechuate and catechol, whereas S-type lignin-derived aromatics degradation occurs via a non-interacting branch (Figure 2) [21–23]. Following lignin depolymerization, aromatics catabolism and ring cleavage, the carbon of these compounds ultimately integrates into tricarboxylic acid cycle.

Table 1. A brief overview of chemical and biological lignin depolymerization methods.

Methods	Advantages	Limitations
Chemical treatments		
Acid catalysts Base catalysts Metallic catalysts Ionic liquids assisted catalysis Supercritical fluids assisted catalysis Oxidative catalysis	Effective lignin degradation High stability Low substrate/product inhibition effects	Low selectivity Environmental concern Costly Difficult recovery of products from the mixture Harsh operational conditions
Biological treatments		
Bacteria Fungi In vitro enzymatic depolymerization	Mild operational conditions Environmentally friendly Optimized by metabolic engineering Optimized by protein engineering Funneling pathways to key intermediates High selectivity and catalytic efficiency	Low conversion yield Long culture/biotransformation time Substrate/product inhibition Difficult to scale up

Biological conversion of lignin is still hampered by the lack of details about the related catabolism pathways and the low conversion ability of microbes. Furthermore, the crude mixture of recovered and pretreated lignin could contain toxic aromatics and inhibiting substances (e.g., sulphite), also showing extreme pH values. Multiple approaches can be relevant in generating a versatile system able to employ a variety of aromatics. Among these approaches are: (i) the search for additional suitable microbial strains; (ii) the determination and recognition of additional lignin degradation pathways; (iii) the synergy between lignin-degrading microbes and novel enzymes: this approach strongly depends on the design/engineering of enzymes with selected properties, e.g., enzymes with improved tolerance, enhanced substrate and cofactor specificity [24,25]; (iv) the use of systems biology to shed light on the metabolic pathways and degradation mechanisms of lignin/aromatics in microorganisms; (v) the systems biology-guided engineering of lignin-degrading microbial strains to build useful strains for utilizing and converting lignin into specific chemicals. The technology platform for targeted and customized strain engineering is quickly growing by incorporating additional elements from synthetic biology, such as libraries of synthetic promoters, ribosomal binding sites and bicistronic elements to allow tunable expression. Furthermore, novel molecular biology toolboxes for DNA synthesis and assembly, as well as targeted and multiplex genome editing allow fast and throughput genetic modifications.

Since the processing of lignin strongly depends on the plant biomass source and the isolation method used, a flexible lignin-degrading strain/approach needs to be generated. Beyond the cellular engineering of existing living organisms, the number of reports focusing on the design, creation and application of synthetic cells are rapidly increasing [26]: such systems aim at the modular programming of genetic circuits and/or synthetic genomes towards a new era of biotechnology.

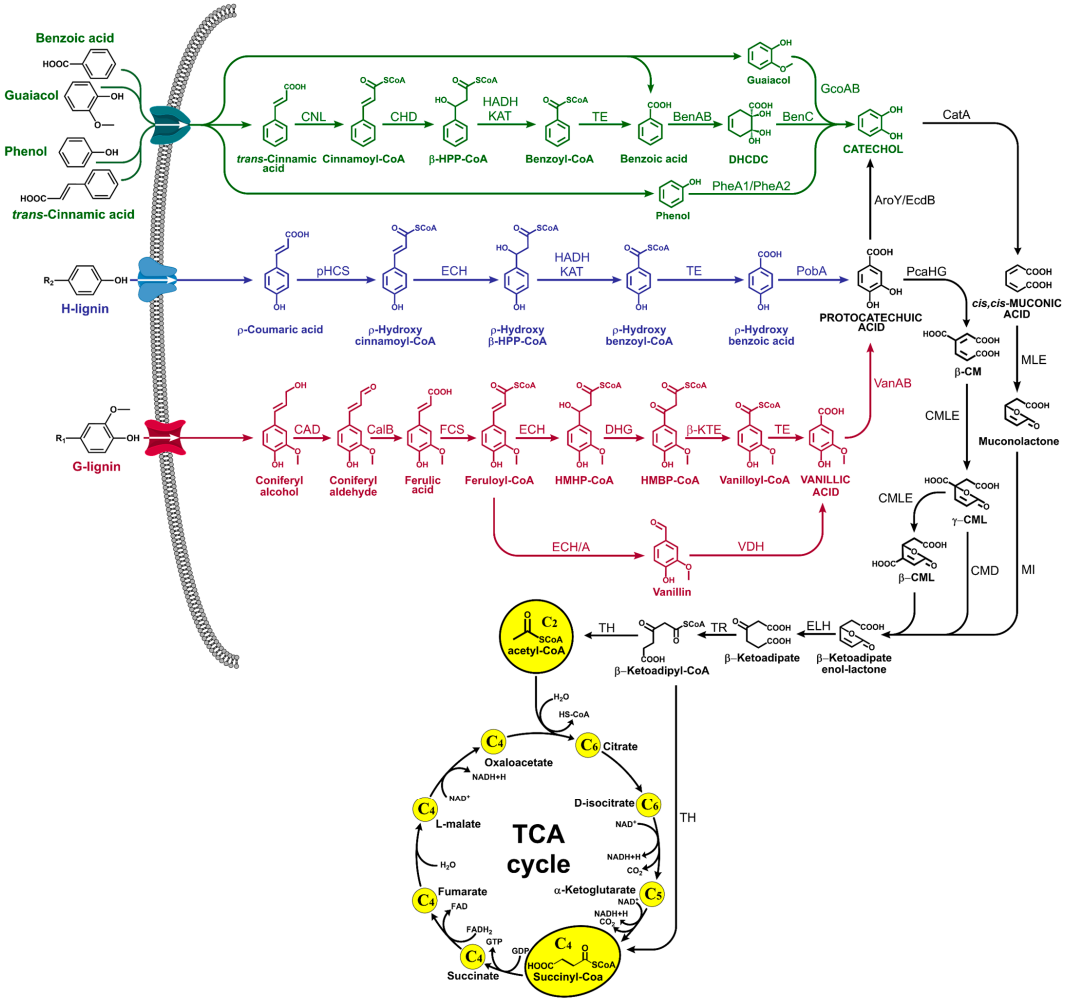

Figure 2. Scheme of the main metabolic pathways involved in the degradation of the aromatic monomers derived from lignin depolymerization. Enzymes involved in the degradation of *trans*-cinnamate, phenol, guaiacol and benzoate (green branch): CNL, cinnamoyl-CoA ligase; CHD, cinnamoyl-CoA hydratase/dehydrogenase; BenA, benzoate 1,2-dioxygenase subunit A; BenB, benzoate 1,2-dioxygenase subunit B; BenC, benzoate 1,2-dioxygenase ferredoxin reductase; BenD, 3,5-cyclohexadiene-1,2-diol-1-carboxylate dehydrogenase; PheA1, large subunit phenol hydroxylase; PheA2, small subunit phenol hydroxylase; GcoA, cytochrome P450; GcoB, cytochrome P450 reductase. Enzymes involved in the conversion of *cis*,*cis*-muconic acid (black branch): CatA, catechol 1,2-dioxygenase; MLE, *cis*,*cis*-muconate lactonase; MI, muconolactone isomerase; PcaHG, protocatechuate 3,4-dioxygenase; CMLE, β-carboxy-*cis*,*cis*-muconate lactonase; CMD, γ-carboxy-muconolactone decarboxylase; ELH, β-ketoadipate enol-lactone hydrolase; TR, β-ketoadipate succinyl-CoA transferase; TH, β-ketoadipyl-CoA thiolase. Enzymes involved in the degradation of H-lignin monomers (blue branch): pHCS, *p*-hydroxycinnamoyl-CoA synthetase; HADH, 3-hydroxyacyl-CoA dehydrogenase; KAT, 3-ketoacyl-CoA thiolase; PobA, *p*-hydroxybenzoate hydroxylase. Enzymes involved in the degradation of G-lignin monomers (red branch): CAD, coniferyl alcohol dehydrogenase; CalB, conyferyl aldehyde dehydrogenase; FCS, feruloyl-CoA synthetase; ECH, enoyl-CoA hydratase; DHG, dehydrogenase; β-KTE, β-keto thiolase; TE, thiolase; ECH/A, enoyl-CoA-hydratase/aldolase; VDH, vanillin dehydrogenase; VanAB, vanillate demethylase A + vanillate *O*-demethylase oxidoreductase; AroY, protocatechuate decarboxylase; EcdB, Ubix family prenyltransferase; HMHP-CoA, 3-(4-hydroxy-3-methoxyphenyl)-3-hydroxypropanoic acid; HMBP-CoA, 4-hydroxy-3-methoxyphenyl-β-ketopropionyl-CoA; β-HPP-CoA, β-hydroxy propionyl-CoA; DHCDC, 1,2-dihydroxy-cyclohexa-3,5-diene-1-carboxylate; β-CM, β-carboxymuconate; β-CML, β-carboxymuconolactone; γ-CML, γ-carboxymuconolactone.

This review highlights recent advances in the field of systems metabolic engineering aimed at exploring the possible conversion of lignin-derived monomers into value-added products, placing much emphasis on the potential practical production of the plastics precursor *cis*,*cis*-muconic acid and of pharmaceutical precursors. The critical review of recent developments of novel biological conversion processes (focusing on their advantages and drawbacks) represents a comprehensive proof of concepts for great potentiality of renewable biomasses-derived aromatics.

Making the whole process of lignin conversion into high-value compounds cost-effective at industrial scale requires the improvement of productivity/yield of the target products. Therefore, the studies discussed in this review, concerning the use of metabolic engineering and systems biology, emphasize that the biological valorization of lignin components into value-added bioproducts is expected to generate considerable market growth and to make the lignin valorization process profitable and sustainable in the near future.

2. Microbial Lignin Depolymerization

Besides its complexity and recalcitrance to degradation, the main problem in industrial lignin valorization is that it is still considered more a problem than a valuable and unexploited resource. All current industrial applications are experimental, pioneering and mainly focused on lignin elimination from the main product mixture. Different strains of white-rot, brown-rot and soft-rot fungi were tested and experimentally used in the bio-pulping process to prepare different vegetal fibrous materials for subsequent chemical and physical treatments in the paper industry. The main drawback is that existing processes still do not match the strict industrial requirements: in most cases the overall process takes days or even months to achieve a suitable degree of delignification [27]. However, in the last decade, several projects not related to bio-pulping were aimed at obtaining valuable chemicals or surfactants directly from lignin [28,29] or lignin-related material, such as advanced biopolymers [30,31].

The first step in lignin microbial degradation is its rough depolymerization performed by extracellular enzymes like peroxidases and laccases. These enzymes cleave unselectively different bonds in lignin, yielding different dimers and monomers. These molecules are small enough to be internalized and funneled through different metabolic pathways (Figure 2).

Both free enzymes and cell fermentation-driven lignin depolymerization suffer the same drawbacks. First of all, enzymatic production is often quantitatively inadequate for industrial purposes: these enzymes are naturally mildly active and expressed at low levels, thus providing limited substrate amounts to sustain the microorganism metabolism. In addition, under ideal operating conditions, wild-type ligninolytic enzymes reach just a fraction of the 10,000 turnovers (moles of product/moles of enzyme) conventionally required as the minimum to economically sustain an industrial bioprocess [32]. Therefore, under harsh pH and temperature conditions and in presence of solvents, the activity of these enzymes decreases very quickly. Moreover, many of these enzymes are subjected to self-deactivation due to heme destruction, suicide inactivation (excess of H_2O_2) and formation of amino acid-based free radicals, yielding a decrease in enzyme reduction potential.

2.1. Microbial Consortia

In nature, lignin depolymerization is often performed by a concert of different organisms, rather than a single one. This usually allows each organism to overcome the deficiency of the others, yielding a quite efficient degradation process. Therefore, one practical strategy to mimic what happens in nature is based on assembling microorganism consortia which may provide a better and faster process. The main problem of this approach is that many microorganisms are still not easily culturable in the laboratory and must be studied using culture-independent strategies, such as metagenomics analyses.

A green fluorescence protein (GFP)-based biosensor was used to detect vanillin and syringaldehyde, which are typical products of lignin metabolism, in high-throughput screening [33]. A total of 147 clones from coal beds were identified for their ability to grow on hardwood and Kraft lignin-enriched media, mostly coming from uncultivable archaea and bacteria. Later on, the same method was used from the aforementioned coal bed sites to identify aromatic transaminases that were able to use lignin-derived monoaromatics producing value-added feedstocks for pharmaceutical synthesis. A novel transaminase, able to aminate as many as 14 monoaromatic lignin-related aldehydes and ketones, was found and characterized [34].

The microbial communities involved in lignin degradation from moderately thermophilic environments such as hot spring sediment (52 °C) and woody "hog fuel" piles (53–62 °C) were studied by a mixed strategy using ^{13}C-ring-labeled synthetic lignin as substrate and monitoring both released $^{13}CO_2$, as a degree of lignin mineralization, and isotopic enrichment of DNA, to identify the genome(s) belonging to lignin-degrading organism(s) [35]. A total of 14 out of 125 draft genomes was enriched in ^{13}C and contained several novel putative laccase-like multi-copper oxidase (LMCO) encoding genes.

Consortia can also be modified and "optimized". To obtain a minimal and effective lignocellulolytic microbial consortium (MELMC), a dilution-to-stimulation/extinction method was implemented using samples from the Andean Forest soil cultured with a mixture of three agricultural wastes. This "dilution-to-stimulation" enriched and stabilized approximately 50 bacterial strains ascribable to the lignocellulolytic component of the original microbial population. Then, this enriched media was inoculated in serial dilutions of the substrate until 10^{-11}, where only the *Pseudomonas* sp. and *Paenibacillus* sp. bacterial species were highly abundant (>99%) [36].

One of the most tricky approaches to consortia is the creation of an artificial one. Consortia can be made up in two ways: (i) top-down, starting from natural consortia and through their characterization and optimization; (ii) bottom-up, trying to establish synergic relationships between different microorganisms not necessarily already involved in a consortium. A valuable example is the method provided by Hu et al. where possible microbial interactions were tested by screening as many microorganisms as possible [37]: several lignocellulosic strains of fungi were tested on a minimal media and the best synergic ligninolytic activities were identified and measured. Then, a screened community enriched in lignin-degrading bacteria was introduced to develop synergic activities with the already-formed fungal consortia. As a result, two fungal (*Trichoderma* and *Aspergillus*) and 16 bacterial strains (from *Bacillus*, *Enterococcus*, *Lactococcus*, *Acinetobacter*, and *Pseudomonas* species) were identified as members of a synergistic microbial consortium showing an improved activity up to 197% [38].

2.2. Extremophilic Microorganisms

The enzymes from extremophiles could represent a suitable alternative in lignin depolymerization to mesophilic bacteria because they already operate in harsh conditions. *Thermobifida fusca* (growing at 45–60 °C) [39] and *Clostridium thermocellum* (growing at 60 °C) [40] are mildly thermophilic bacteria which show or express thermophilic enzymes with detectable lignin depolymerization activity. At higher temperatures, fewer bacteria are able to use lignin. As an example, *Archaeoglobus fulgidus* (growing at 80 °C) is suspected to use lignin-derived substrates based on a recently discovered methoxydotrophic metabolism, which acts on methoxylated aromatic molecules [41] quite common in lignin structure. Similarly, a laccase from the hyperthermophilic *Thermus thermophilus* strain showed an optimum of temperature at 90 °C [42].

On the other hand, several microorganisms show a remarkable lignin degradation activity at low temperatures, this offering advantages for biotechnological processes and for low-energy treatments. Several psychrophilic microorganisms were identified from different environments, both fungi such as *Rhodosporidiobolus colostri* from the alpine forest (1–25 °C) [43] and various Antarctic filamentous fungi from King George Island in

Antarctica (growing at 1–15 °C) [44], and bacteria, such as *Arthrobacter* sp. from Chinese Manchuria (growing at 15 °C) [45] and *Burkholderia* sp. from Alaskan tundra soil (growing at 15 °C) [46]. Recently, from the marine *Halomonas* sp. strain M68 isolated from marine biofilm and water samples collected in Terra Nova Bay (Antarctica), an intracellular thermo- and halo-tolerant laccase-like activity, with an optimal temperature in the 40–50 °C range, was isolated [47].

Halophiles bacteria are quite interesting due to the uncommon resistance to high saline levels, a feature usually linked to remarkable tolerance to UV light radiations, temperature, and extreme pH values. Actually, *Penicillium chrysogenum*, isolated in saline soil, has the capability to depolymerize lignin directly on olive milled substrate from oil production [48]; meanwhile, several halophilic ligninolytic enzymes were isolated and tested on raw substrates such as sugar beet pulp [49], almond shells [50] or peanut shells [51]. All these microorganisms, and related enzymes, were tested successfully on such substrates with minimal or no pre-treatment.

Significantly, several laccases were identified as solvent-resistant [19,52]. Besides only a few of them that were tested and characterized, both fungi [53] and bacteria [54] proved to be a promising source of solvent-tolerant enzymes, particularly useful to treat high-molecular weight lignin polymers, usually scarcely soluble in aqueous media [13].

3. Lignin Valorization: The Biological Routes towards Value-Added and Specialty Chemicals

Microorganisms show the ability to convert lignin, as well as lignin-derived compounds, into several value-added molecules, see Table 2 [55–62]. Regardless, due to the mentioned heterogeneity of lignin, its depolymerization results in the production of a number of monomeric aromatic compounds such as vanillin, guaiacol, catechol and protocatechuic acid (PCA) (Figure 2): their funneling into targeted chemicals of industrial value (e.g., bio-based plastic precursors as *cis*,*cis*-muconic acid, ccMA), is crucial for an efficient biomass valorization. The biological route approach to lignin valorization is very promising, mainly due to the abundant biochemical pathways present in nature [63,64], or assembled in the laboratory [65,66].

Table 2. Value-added compounds obtained from lignin or lignin-derived aromatics by microbial degradation pathways.

Product	Host	Starting Material	Yield	Reference
Coniferyl alcohol	*Amycolatopsis* sp. HR167 pRLE6SK*vaom*	Eugenol	4.7 g/L	[55]
Ferulate	*E. coli* XL-1 Blue pSK*vaomPcalAmcalB*	Eugenol	14.7 g/L	[56]
Lactate	*P. putida* KT2440-CJ127 *P. putida* KT2440-CJ124	Benzoate *p*-Coumarate	0.5 g/L 0.5 g/L	[57] [57]
PCA	*P. putida* KT2440	Ethanol-assisted depolymerized lignin	6.7 mg/L	[58]
Pyruvate	*P. putida* KT2440-CJ112 *P. putida* KT2440-CJ116	Benzoate *p*-Coumarate	0.7 g/L 2 g/L	[57] [57]
Succinate	*Phanerochaete chrysosporium*	Lignin	20 mg/L	[59]
Vanillin	*R. jostii* RHA045 *Shewanella putrefaciens* *Pseudomonas* sp. HR199 Δ*vdh* *E. coli* pSK*echE*/*Hfcs* *Streptomyces setonii*	Wheat straw lignocellulose Lignin from wheat straw Eugenol Ferulate Ferulate	96 mg/L 275 mg/L 400 mg/L 300 mg/L 6.4 g/L	[60] [61] [62] [56] [67]

The most investigated metabolic routes employ upper pathways to drive aromatic compounds into the β-ketoadipate pathway via the catechol node, a central hub of aromatic catabolism ("biological funneling approach"): many aromatic-catabolizing microorganisms employ a catechol-1,2-dioxygenase (CatA) enzyme to open the catechol ring to produce

ccMA [23]. Moreover, different aromatics from guaiacyl- and *p*-hydroxyphenyl-units of lignin are catabolized into PCA and further metabolized into ccMA (see Figure 2).

3.1. Cis,cis-Muconic Acid

ccMA is a dicarboxylic acid of recognized industrial value [68,69]: it represents a potential platform chemical for the synthesis of a variety of polymers (polyamides, polyethylenes, and polyurethanes) [70–72]. ccMA can be easily hydrogenated into adipic acid, a building block of commercial nylons and polyurethanes, or converted into terephtalic acid (TPA), one of the two main monomeric components of the high-demand polyethylene terephthalate (PET) [70,73]. It has been estimated that the global market for ccMA derivatives is USD 22 billion [74], with a market for ccMA alone at more than USD 100 million [72].

Traditionally, ccMA has been chemically produced using petroleum-based feedstocks and high concentrations of heavy metal catalysts, thus generating toxic intermediates with heavy environmental pollution. Moreover, the chemical process produces a mixture of muconic acid enantiomers (i.e., ccMA and *cis,trans*-muconic acid), thus requiring downstream purification processes and additional costs [74]. Therefore, the production of ccMA from renewable sources such as lignocellulosic biomass represents a suitable and appealing green alternative.

The current biological production of ccMA is mainly focused on the introduction in the selected microorganisms of synthetic pathways to convert the intermediates metabolites of the natural shikimate pathway into ccMA starting from glucose [73,75,76]. Significantly, while sugars derived from biomass pre-treatment are in high demand for food, lignin-derived aromatics represent a feedstock not competing with the food chain also available at a lower cost than sugars. Since the 1970s, the biobased production of ccMA from benzoic acid and toluene has been reported, reaching a >93% yield (moles of ccMA per moles of benzoic acid) [77], but environmental concerns pushed towards renewable feedstocks [78,79]. The engineered strains such as *Amycolatopsis* sp. [80], *Corynebacterium glutamicum* [81,82], *Rhodococcus opacus* [83,84], *Escherichia coli* [74,85–87], and *Pseudomonas putida* KT2440 [23,88–94] have been reported to produce high ccMA titers from lignin-related aromatics. As a general rule, different systems biocatalysis approaches rely on the blockage of ccMA degradation pathways to favor its accumulation and on the overexpression of heterologous enzymes involved in the conversion of lignin-derived aromatics into ccMA. A summary of engineering strategies adopted for constructing optimized ccMA-producing strains is reported in Table 3.

3.1.1. *Amycolatopsis* sp. ATCC 39166

Amycolatopsis sp. ATCC 39166 has been reported as one of the few microorganisms able to utilize guaiacol in a metabolic pathway involving its demethylation into catechol, a precursor of ccMA [95]. A tailored genomic modification of the actinomycete *Amycolatopsis* sp. ATCC 39166 has been successfully developed by homologous recombination to produce ccMA from guaiacol. By deletion of two genes (AATC3_020100018510, 1104 bp and AATC3_020100009302, 1278 bp) encoding for putative muconate cycloisomerases (CatB) involved in the β-ketoadipate pathway, the MA-2 mutant strain was generated (Figure 3A): the optimized engineered strain produces 3.1 g/L ccMA from guaiacol after 24 h incubation, with a 96% yield, when cultivated at 37 °C in a fed-batch process using a stirred tank bioreactor initially supplemented with 5 mM guaiacol and 6 g/L glucose, followed by a pulse-wise guaiacol addition (Table 3).

Interestingly, when the hydrothermal depolymerization of an industrial softwood Kraft lignin from pine (Indulin AT) was conducted for 20 min at 330 °C, four most abundant aromatics were generated with a 12% conversion yield containing guaiacol (44%), catechol (30%), phenol (14%), and *o*-cresol (12%). The additional distillation step enriched the lignin hydrolysate of guaiacol: the total process provided an aqueous solution containing 7 g/L guaiacol, 3 g/L *o*-cresol, and 0.2 g/L phenol [80]. Significantly, starting from this lignin hydrolysate, the optimized MA-2 strain consumed all aromatics (2.5 mM) within about

10 h generating 1.8 mM ccMA and improving up to 6-fold the production efficiency in comparison to the wild-type strain (Table 3).

Table 3. Metabolic engineering strategies for constructing optimized ccMA-producing strains.

Host	Starting Material	Deleted Genes	Overexpressed Genes	Productivity (g/L/h)	Reference
Amycolatopsis sp. ATCC 39166	Guaiacol Hydrothermal depolymerized Kraft lignin	*G10GW-3575, G10GW-1735* (putative cycloisomerases)	-	0.13 0.26	[80]
C. glutamicum	Catechol Hydrothermal depolymerized Kraft lignin	*catB*	*catA*	2.4 0.07	[82]
R. opacus	PCA Lignin from corn stover	*catB, pcaHG*	*aroY, ecdB*	0.56 0.02	[83]
	Vanillin	*LPD03722, LPD04406* (vanillin reductases) *pcaHG*	*vdh, aroY, ecdB*	0.22	[84]
E. coli	Vanillin	-	*vdh, vanAB, catA, aroY, kpdB*	0.04	[85]
			ligV, ligM, aroY, catA	0.01	[74]
			ligV, vanAB, aroY, catA	0.7	[86]
	Vanillin from lignin	-	*ligV, vanAB, aroY, catA*	0.3	
	Isoeugenol	-	*ado, hfd1, vanAB, gdc, catA*	0.14	[87]
	Ferulic acid Ferulic acid from wheat bran	-	*fdc, ado, ligV, vanAB, aroY, catA*	0.14 0.04	[86]
P. putida	*p*-Coumarate Alkaline pretreated liquor	*pcaHG, catBC, dmpKMLNOP*	*aroY*	0.17 0.03	[88]
	p-Coumarate Ferulic acid	*pcaHG, catRBCA, crc*	*catA, aroy*	0.08 0.01	[90]
	p-Coumarate	*pobA, catRBCA, crc*	*praI, ecdBD, vanAB*	0.4	[91]
	Catechol Hydrothermal depolymerized Kraft lignin	*catBC, endA-1, endA-2*	*catA* *catA, dmpKLMOP*	4.3 0.24	[92]
	Vanillic acid	*pcaHG, catB*	*pdc*	0.03	[94]
	Vanillic acid Sugar cane bagasse alkaline extract	*catB*	*pcaHG, aroY, catA. vanAB*	0.21 0.01	[93]

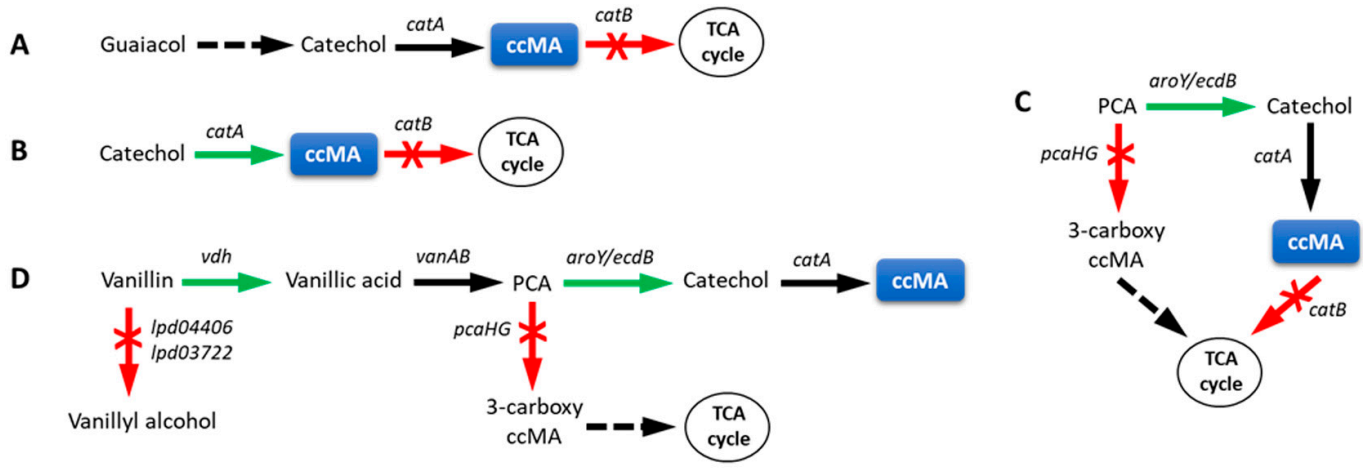

Figure 3. Scheme of ccMA production from engineered (**A**) *Amycolatopsis* sp. ATCC 39166 [80], (**B**) *Corynebacterium glutamicum* [82], and (**C**,**D**) *Rhodococcus opacus* [83,84]. Encoding genes: *aroY*, protocatechuate decarboxylase; *catA*, catechol 1,2-dioxygenase; *catB*, muconate cycloisomerase; *ecdB*, flavin prenyltransferase; *lpd03722* and *lpd04406*, putative vanillin reductases; *pcaHG*, protocatechuate 3,4-dioxygenase; *vdh*, vanillin dehydrogenase; *vanAB*, vanillate demethylase. Black arrows show the endogenous pathway; green arrows show overexpressed pathways; red crossed arrows identify deleted pathways; dashed arrows show additional multi-step pathways.

3.1.2. *Corynebacterium glutamicum*

Similar to the genetic modification reported above for *Amycolatopsis* sp ATCC 39166, a metabolic engineering strategy to upgrade the ccMA production has been recently carried out for the soil bacterium *C. glutamicum* ATCC 13032 [82]. This microorganism is able to degrade a wide spectrum of aromatic compounds via the β-ketoadipate pathway, representing the main assimilation pathway of lignin-derived compounds [22].

A stable genome manipulation by using the integrative, non-replicating plasmid pClik in sacB [81] was carried out at the level of the catechol branch of the β-ketoadipate pathway: an engineered strain deleted of the gene *catB* (NCgl2318) encoding the muconate cycloisomerase enzyme (to block the catabolism of ccMA) was established (*C. glutamicum* MA-1). When cultivated in shake flasks at 30 °C, 5 mM phenol, 10 mM catechol, and 20 mM benzoic acid have been fully converted into ccMA in 24 h. In addition, to enable faster ccMA production, the targeted overexpression of catechol-1,2-dioxygenase (CatA), catalyzing the intradiolic opening of the aromatic ring of catechol to give ccMA was established by using the strong constitutive P_{tuf} promoter (NCgl0480): for the obtained strain (*C. glutamicum* MA-2), a 10-fold higher CatA expression level was achieved in comparison to the single mutant strain *C. glutamicum* MA-1 [82] (Figure 3B). The constitutive expression of the *catA* gene (NCgl2319) reached a ccMA production rate of 5.2 mmol g^{-1} h^{-1} starting from catechol, a value 25-fold higher than that observed for the non-induced strain, with a full molar yield. Moreover, in the fed-batch process using pulse-wise feeding of catechol (to avoid toxic levels) and of glucose (to maintain its level in the 5–15 g/L range), the optimized *C. glutamicum* MA-2 strain accumulated up to 85 g/L ccMA after 60 h of incubation, with a maximum volumetric productivity of 2.4 g L^{-1} h^{-1} (Table 3). The performance of the established strain was also evaluated on real lignin samples: the hydrothermal treatment of the softwood Kraft lignin from pine (Indulin AT) was carried out in supercritical water, resulting in the enrichment of the liquid phase in catechol (101 mM) and phenol (25 mM). The lignin hydrolysate, supplemented with glucose, was added as pulse to the *C. glutamicum* MA-2 culture grown in minimal medium in baffled shake flasks: a production of 1.8 g of ccMA per liter of broth after 27 h of incubation was achieved (corresponding to 0.07 g L^{-1} h^{-1}, Table 3).

3.1.3. *Rhodococcus opacus*

R. opacus PD630 is a Gram-positive bacterium well suited for lignin valorization mainly because of its high tolerance to lignin-derived breakdown compounds coupled to the ability to catabolize a number of aromatics [87–89]. Recently, *R. opacus* has been engineered for the first time to produce ccMA from lignin-derived monomeric aromatic compounds and real lignin samples, towards the catechol and PCA funneling nodes (Figure 2), blocking the related degradation pathways [74]. A number of *R. opacus* mutants has been successfully established by using a gene deletion/insertion strategy based on a mutant phenylalanyl-tRNA synthase gene (pheS*) as a counter-selection marker in the second homologous recombination, and with the *p*-Cl-phenylalanine as a screening factor. Briefly, when the pheS* expression cassette is integrated into the *R. opacus* PD630 genome, the recombinant strain will be sensitive to *p*-Cl-Phe, whereas mutants loosing the pheS* expression cassette will recover *p*-Cl-Phe resistance [90]. Based on the genome analysis of *R. opacus* PD630, at first the gene encoding for the muconate cycloisomerase CatB (Pd630_LPD06567) has been deleted (to block the ccMA degradation in the β-ketoadipate pathway), by using the suicide plasmid pK18mob-pheS * yielding the *R. opacus* PD630-MA1 strain. When 10 mM catechol was supplied, the engineered *R. opacus* PD630-MA1 strain generated 9.7 mM ccMA (Table 3). Analogously, to block the PCA degradation pathway, the genes encoding the α- (Pd630_LPD05451) and β-subunit (Pd630_LPD05450) of protocatechuate 3,4-dioxygenase PcaHG in the *R. opacus* PD630-MA2 mutant have been deleted generating the *R. opacus* PD630-MA4 mutant. Simultaneously, to push protocatechuate into the catechol degradation pathway, the genes encoding protocatechuate decarboxylase AroY (GenBank: ADF61496) and UbiX-like prenyltransferase EcdB (GenBank: ADF63617) from *Enterobacter cloacae* have

been inserted in the endogenous plasmid of the PD630-MA4 strain, thus generating the PD630-MA6 mutant (Figure 3C). The established strain accumulated ccMA from PCA with a yield of 0.91 mol/mol (Table 3) [74]. Significantly, when the *R. opacus* PD630-MA6 mutant was incubated with a commercial alkali lignin pretreated with 1% (*w*/*w*) H_2SO_4 and 1% (*w*/*w*) NaOH at 120 °C for 30 min, and added to the commercial laccase from *Aspergillus*, 1.63 g/L ccMA was produced by a fed-batch fermentation process after 72 h incubation (Table 3) [74].

Interestingly, the engineered strain converted several lignin-derived aromatics (*p*-coumarate, vanillin, vanillyl alcohol, vanillate, coniferyl alcohol, ferulate, *p*-hydroxybenzoate, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzyl alcohol) into ccMA: after incubation with 10 mM of each substrate, their concentration decreased up to 2–5 mM. In particular, the observation that only 10% vanillin and 4% *p*-hydroxybenzaldehyde (mol/mol) were converted into ccMA indicates that alternative metabolic pathways for these aromatic aldehydes are active, e.g., vanillin was mainly converted into vanillyl alcohol [75]. A total of 23 potential vanillin reductases were predicted by BLASTp analysis, using the sequence of the verified enzyme from *Pseudomonas* sp. strain 9.1 (FEZ21_09870) [91], and their transcriptional level assessed by qRT-PCR [75]. The 11 selected putative vanillin reductases have been expressed in *E. coli* BL21 cells to evaluate their ability to convert vanillin into vanillyl alcohol: the enzymes encoded by the genes *Pd630_LPD03722* and *Pd630_LPD04406* showed the highest conversion yield. Accordingly, a "reducing expenditure and broadening sources" strategy has been adopted to engineer the *R. opacus* PD630-MA6 strain, see above. The mentioned genes encoding putative vanillin reductases were deleted from the *R. opacus* PD630-MA6 mutant, and the gene encoding vanillin dehydrogenase Vdh from *Sphingobium* sp. SYK-6 (BAK65381.1) was overexpressed, to draw the vanillin metabolic flux towards ccMA [75] (Figure 3D). Notably, the vanillyl alcohol production decreased from 85.6% to 1.7%, thus enhancing the ccMA production up to a 97.8% yield after 24 h of incubation, in comparison to the starting 10% (mol/mol) bioconversion yield (Table 3).

3.1.4. *Escherichia coli*

E. coli represents a promising cell factory for ccMA production, mainly due to its incomparable fast growth and established gene manipulations tools available for its metabolic engineering. The first report on the ccMA production from lignin-derived aromatics via a biosynthetic pathway established in *E. coli* relies on the conversion of vanillin into ccMA by the mixture of *E. coli* XL-1blue/pHM002 and pTS052 recombinant strains [85]. The whole cell system has been co-transformed with the pHM002 plasmid encoding vanillin dehydrogenase (Vdh), the α- and β-subunits of vanillate demethylase (VanAB), and catechol dioxygenase (CatA) from *P. putida* NBRC100650, and the plasmid pTS052 encoding protocatechuate decarboxylase (AroY) from *Klebsiella pneumoniae* A170-10 and the β-subunit of the 4-hydroxybenzoate decarboxylase (KpdB) from *K. pneumoniae* NBRC14940, to improve the PCA decarboxylase activity (reaching a 14-fold increase compared to the strain expressing the AroY enzyme alone) [85]. The resting cells have been incubated at 30 °C under shaking in 1 mL of M9 medium containing 1 mM vanillin: a ≈ 80% bioconversion yield was achieved after 3 h of incubation with vanillic acid accumulation, probably due to the ccMA feedback inhibition of the demethylase activity (Table 3).

Later on, the valorization of lignin-derived vanillin has been demonstrated by a hybrid biochemical route combining alkali lignin depolymerization and the reconstruction of the heterologous synthetic pathway in the *E. coli* DH1 strain [74]. Vanillin has been obtained by oxidation of Kraft lignin added to hydrogen peroxide as a catalyst, with a yield in the range of 3–5% (*w*/*w*), and used as substrate for the bioconversion reaction. To accomplish the bioconversion of vanillin into ccMA, the genes encoding vanillin dehydrogenase (LigV), 3-*O*-methylgallate-*O*-demethylase (LigM) from *Sphyngobium* sp. SYK-6 [66,96], and protocatechuate decarboxylase (AroY) [66] from *K. pneumoniae* were stacked in a single operon in the pBbE1a plasmid under the control of the P_{trc} promoter, while the gene encoding catechol 1,2-dioxygenase (CatApmt2) from *P. putida* mt-2 was cloned in the pBbE1 plasmid under

the control of the T7 promoter. All genes were codon optimized for expression in *E. coli*. The whole cells harboring both plasmids were harvested, resuspended in M9 medium added of 10 g/L glucose and 0.5 g/L vanillin in a 5% of the original volume, and incubated under shaking at 30 °C: a bioconversion yield of 341 mg/L ccMA was achieved, corresponding to 0.69 g of ccMA produced from 1 g vanillin, without accumulation of intermediate metabolites in the reaction mixture [74] (Table 3). The whole cell platform expressing the synthetic pathway under the isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducer promoter has been further optimized by the successful integration of an autoregulatory vanillin-inducible system and the co-expression of an active aromatic transporter [97]. In detail, the biocatalytic pathway is under induction of the vanillin self-inducible promoter ADH7 from *Saccharomyces cerevisiae* [98], and at the same time, vanillin is efficiently transported across cell membranes by co-expressing the CouP transporter of the ABC system from *Rhodopseudomonas palustris* (under the control of the ADH7 promoter), showing a dissociation constant for phenylpropanoid ligands in the nanomolar range [99]. This strain showed a 40% increase in catechol production compared to the one not expressing CouP.

The engineered *E. coli* system represents an appealing route for lignin valorization notwithstanding the cost (and often the toxicity) of inducers [100], such as IPTG. Moreover, different lignin-derived aromatics present in lignin depolymerization mixtures have been reported to inhibit the enzyme activity and cell growth [101,102]: a self-regulation system could reduce the "toxicity" of aromatics such as vanillin, which can be therefore used as both inducer and substrate.

A useful strategy to improve the bioconversion yield, and in particular to reduce the bottlenecks represented by alternative biosynthetic routes, is represented by the engineered *E. coli* K-12 MG1655 RARE [103], a strain showing low aromatic aldehyde reduction activities and thus aimed at preventing the conversion of vanillin into the by-product vanillyl alcohol [66]. A whole cell biocatalyst to convert isoeugenol into ccMA has been developed using the engineered strain expressing aromatic dioxygenase (Ado) from *Thermothelomyces thermophila* [104], aldehyde dehydrogenase (Hfd1) from *S. cerevisiae* [105], vanillic acid *O*-demethylase (VanAB) from *P. putida* KT2440 [94], gallate decarboxylase (Gdc) from *Talaromyces atroroseus*, and catechol dioxygenase (CatA) from *Acinetobacter radioresistens* [106] (Figure 4A). The whole cell biocatalyst expressing Ado and Hfd1 enzymes almost completely converted 10 mM isoeugenol into vanillic acid [87]. When the engineered strain expressing the overall synthetic pathway was fed with 10 mM isoeugenol, 2.1 mM ccMA only was produced, with accumulation of vanillic acid, thus suggesting inhibition of VanAB activity by isoeugenol. This bottleneck was overcome by a two-step approach combined with a vanillic acid pulse-feeding strategy (i.e., by adding 10 mM vanillic acid every 90 min) (Figure 4A): 25 mM isoeugenol-derived vanillic acid has been converted into 23.3 mM ccMA after 24 h of incubation, with a 93.1% conversion yield (Table 3) [87]. Significantly, a low conversion rate was observed when the vanillic acid concentration was increased up to 50 mM because of the drop in the pH value of the reaction mixture due to the ccMA accumulation: pH control at 8.0 resulted in the complete consumption of vanillic acid and a 87.6% conversion yield into ccMA.

Very recently, the *E. coli* K-12 MG1655 RARE strain has been employed to convert in one-pot both vanillin arising from lignin and ferulic acid isolated from wheat bran into ccMA [86]. The whole cell biocatalyst has been engineered with three plasmids differing in copy numbers to modulate the expression of seven recombinant enzymes to convert vanillin into ccMA: the phenolic acid decarboxylase Fdc from *Bacillus pumilus* and the aromatic dioxygenase Ado from *T. thermophila* to convert ferulic acid into vanillin, and the vanillin dehydrogenase LigV from *Sphingobium* sp. SYK-6, the vanillate *O*-demethylase oxygenase VanAB, the protocatechuate decarboxylase AroY from *Klebsiella pneumonia* subsp., and the catechol dioxygenase from *Acinetobacter radioresistens* S13 (Figure 4B). The optimization of the extraction procedures for vanillin from lignin and of ferulic acid from wheat bran, combined with selected reaction conditions of the whole cell biocatalyst, allowed the production of ccMA from lignin-derived vanillin in one-pot with a >95% conversion

yield in 30 min and a productivity of 4.2 mg of ccMA per g of Kraft lignin. Moreover, the optimized whole-cell system expressing all the above-mentioned enzymes produced 2.2 mg of ccMA/g of wheat bran in 10 h, with a >95% conversion yield (Table 3) [86].

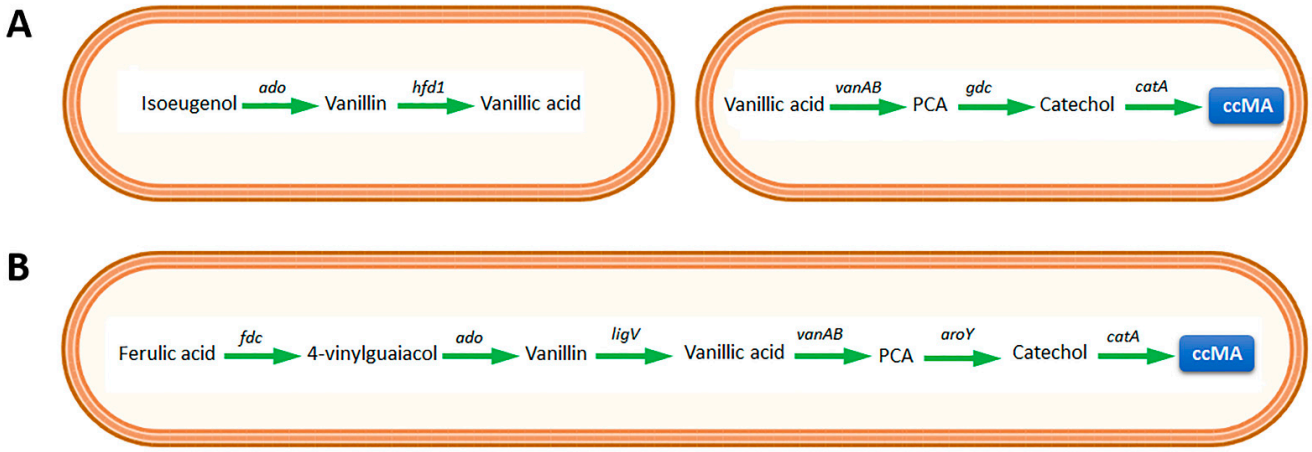

Figure 4. Synthesis of ccMA from (**A**) isoeugenol in a two-step approach [87], and from (**B**) ferulic acid in one-pot [86] using engineered *Escherichia coli* strains. Encoding genes: *ado*, aromatic dioxygenase; *aroY*, protocatechuate decarboxylase; *catA*, catechol 1,2-dioxygenase; *fdc*, phenolic acid decarboxylase; *gdc*, gallate decarboxylase; *hfd1*, aldehyde dehydrogenase; *ligV*, vanillin dehydrogenase; *vanAB*, vanillate demethylase. Green arrows show overexpressed pathways.

3.1.5. *Pseudomonas putida*

P. putida KT2440 is able to catabolize a number of lignin-derived aromatics, thus representing a native ccMA producer. The known genetic manipulation strategies, combined with the ability to tolerate a variety of physical and chemical stresses, made this microorganism the most used cell factory system to produce ccMA from many aromatics. *P. putida* KT2440 has been metabolically engineered to convert a number of lignin-derived aromatics such as ferulic acid, vanillin, coniferyl alcohol and *p*-coumarate into ccMA through both the catechol and PCA branches of the β-ketoadipate pathway (Figure 2) [88]. In detail, the *pcaHG* gene encoding the protocatechuate 3,4-dioxygenase was replaced by the *aroY* gene for the PCA decarboxylase from *Enterobacter cloacae*, in order to funnel the PCA metabolism toward the production of catechol and block the conversion of PCA into β-ketoadipate. To enable the constitutive expression of the catechol 1,2-dioxygenase CatA enzyme, to avoid further ccMA conversion and to expand substrate utilization (e.g., including phenol, a commonly-derived lignin degradation intermediate), the genome region corresponding to genes *catR*, *catBC*, and to promoter for *catBCA* were replaced with the P_{tac} promoter, and the genes *dmpKLMNOP* encoding the phenol monooxygenase from *Pseudomonas* sp. CF600 [107] were integrated downstream the *catA* gene under the tac promoter (Figure 5). The optimized engineered *P. putida* KT2440-CJ103 strain successfully converted 10 mM catechol, phenol and benzoate (via the catechol node) and 10 mM PCA, coniferyl alcohol, ferulic acid, vanillin, caffeic acid, *p*-coumarate and 4-hydroxybenzoic acid (4-HBA) (via the PCA node) into ccMA, using acetate as carbon and energy source, with a yield in the 14–93% range. A significant vanillic acid accumulation resulted starting from vanillin, ferulic acid and coniferyl alcohol, probably ascribable to the mentioned transcriptional regulation strategies. The engineered strain was used in a fed-batch bioreactor with aeration and pH control: a ccMA titer of 13.5 g/L was achieved after 78.5 h of incubation using *p*-coumarate as substrate (2 mM), a value 15-fold higher than the bioconversion yield obtained in shake flasks cultivation [88]. Interestingly, the *P. putida* KT2440-CJ103 strain produced 0.7 g/L of ccMA after 24 h of incubation from an alkaline liquor stream obtained by the corn stover pre-treatment with NaOH and antraquinone (Table 3). Considering that ferulic acid and *p*-coumarate were initially present as the two major aromatics (0.34

and 0.92 g/L, respectively), a 67% molar yield was achieved. Significantly, following the addition of activated carbon (12.5% wt/vol) to the culture media under stirring for 1 h to remove oxygenated aromatics, ccMA was precipitated by reducing the pH and temperature values and crystals recovered by vacuum filtration: a 74% recovery yield was obtained, with a >97% degree of purity.

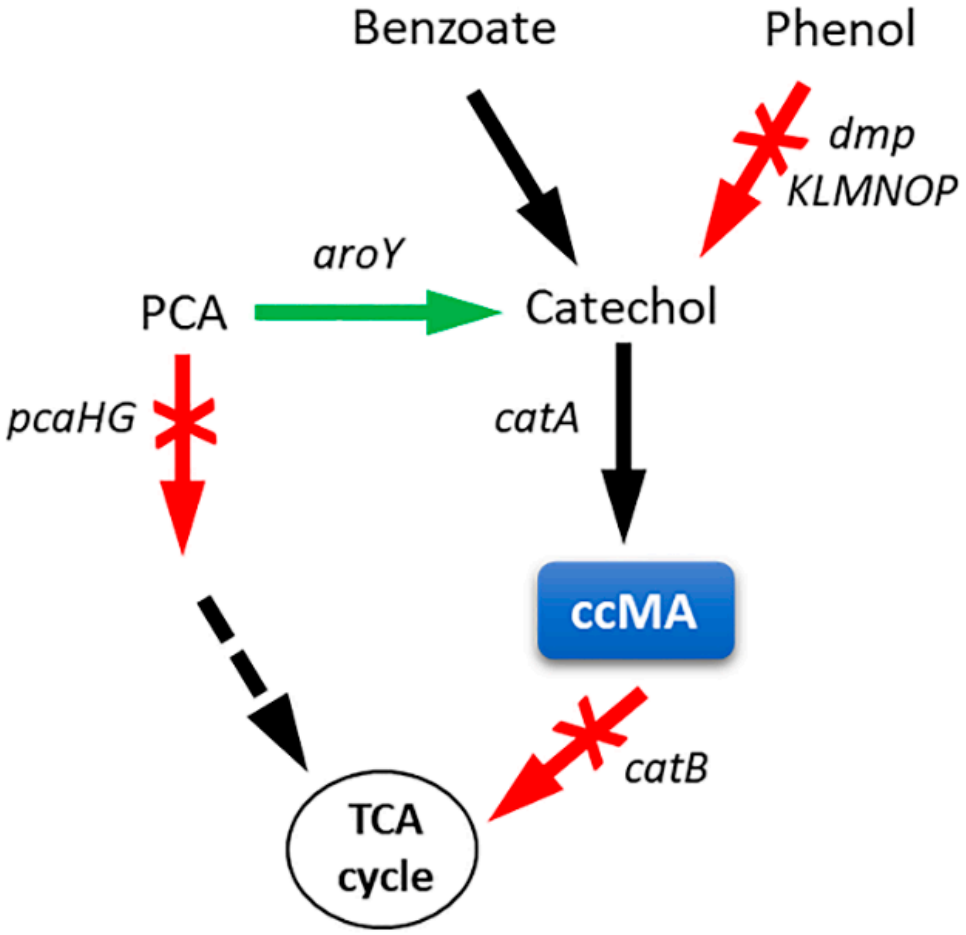

Figure 5. Metabolic engineering of *Pseudomonas putida* KT2440 to convert protocatechuic acid (PCA) and catechol derived from benzoate and phenol into ccMA [88]. Encoding genes: *aroY*, protocatechuate decarboxylase; *catA*, catechol 1,2-dioxygenase; *catB*, muconate cycloisomerase; *dmpKLMNOP*, phenol monooxygenase; *pcaHG*, protocatechuate 3,4-dioxygenase. Black arrows show the endogenous pathway; green arrows show overexpressed pathways; red crossed arrows identify deleted pathways; dashed arrows show additional multi-step pathways.

It is well known that many aromatic degradation pathways involved in the ccMA production are controlled by transcriptional and translational regulators, resulting in the accumulation of intermediates such as vanillate, PCA and 4-HBA, thus reducing the productivity of the overall process [88,108]. In *Pseudomonas*, the most important of these mechanisms is controlled by the Crc (catabolite repression control) protein, a translational regulator that inhibits both the transcription of mRNA encoding transcriptional regulators of catabolic enzymes and transporters of substrates inside cells [89]. By a mass spectrometry-based proteomics investigation, the 4-HBA hydroxylase PobA enzyme and the vanillic acid demethylase VanAB enzymes (involved in the bioconversion of 4-HBA and vanillic acid into PCA) have been identified as targets of the Crc protein [90]: the deletion of the *crc* gene led to enhanced ccMA production from ferulic acid and *p*-coumarate when glucose or acetate were used as carbon and energy sources [90]. The resulting *P. putida* KT2440-CJ238 engineered strain was grown on 10 mM glucose or 30 mM sodium acetate (with

additional feeding every 12 h to provide energy and carbon for growth) in the presence of 20 mM ferulic acid or *p*-coumarate as substrates. In cultures grown on glucose, the ccMA was obtained with a yield of 94.6 ± 0.6% (mol/mol) after 36 h of incubation in comparison to a 56.0 ± 0.3% (mol/mol) figure observed for the parental strain (Table 3). Similarly, a faster metabolism of the vanillate intermediate was apparent using ferulic acid as substrate: after 72 h of incubation, ccMA was produced by the engineered KT2440-CJ238 strain with a 28.3 ± 3.3% (mol/mol) yield, compared to 12.0 ± 2.3% (mol/mol) for the KT2440-CJ102 parental strain. Less pronounced effects were observed when acetate was used as a cost-effective alternative substrate, with a 47.7 ± 0.6% (mol/mol) yield after 24 h from *p*-coumarate and a 16.9 ± 1.4% (mol/mol) yield after 72 h from ferulic acid [90].

The bottleneck represented by the rate-limiting activity of the 4-HBA hydroxylase has been also alleviated by the engineering of the *P. putida* KT2440 strain expressing an alternative hydroxylase enzyme: the endogenous gene encoding PobA (a NADPH obligate enzyme) has been replaced by the *praI* gene encoding the enzyme from *Paenibacillus* sp. JJ-1b, which utilizes either NADH and NADPH as cofactor [91]. The engineered CJ475 strain with enhanced activity of the protocatechuate decarboxylase AroY through the co-expression of EcdBD, deleted of a global regulator of carbon catabolite repression and of the *catRBCA* gene, overexpressing the native vanillate *O*-demethylase VanAB [109], has been further engineered by integrating the *praI* gene into the bacterial genome and deleting the *pobA* gene (CJ781 strain) [91]. In a bioreactor cultivation system, with a feed of 6 mmol of *p*-coumarate per hour, a ccMA titer of 43 g/L was achieved with a 96 mol% yield, and a productivity of 0.4 g L^{-1} h^{-1} (Table 3).

A useful metabolic strategy to increase the catechol tolerance has been reported by [92]. The engineered *P. putida* KT2440-M6 strain was constructed by deleting the *catBC* gene, as reported before for the KT2440-CJ103 strain [88], and two endonuclease encoding genes *endA-1* and *endA-2*, and by boosting the expression of the catechol 1,2-dioxygenase CatA by the insertion of a second genomic copy of the *catA2* gene downstream of the *catA* gene under the control of the native P_{cat} promoter [92]. The engineered strain showed a 20% improvement in catechol tolerance, thus allowing the production of 64.2 g/L ccMA from catechol after 15 h of incubation in a fed batch process, with a productivity of 4.3 g L^{-1} h^{-1} (Table 3).

As reported before for the engineered *P. putida* KT2440-CJ103 strain [88], and with the aim to expand the substrate spectrum to cresols, the gene *dmpKLMOP* encoding the phenol hydroxylase from *P. putida* CF600 was inserted into the genome of the KT2440-M6 strain, designated as M9 strain. The engineered biocatalyst successfully converted the hydrolysate obtained by the hydrothermal treatment of softwood lignin in supercritical water (which mainly contained catechol, phenol and small amounts of *p*-cresol and *o*-cresol) into ccMA with a titer of 13 g/L after 54 h of incubation (Table 3). After two treatments of the reaction mixture with active carbon, followed by crystallization and lyophilization, a dry powder containing 96.3% ccMA was obtained. The established process has been successfully scaled up to a 50-L pilot scale reactor: maintaining the concentration of catechol below 2 mM by a pulse-feeding strategy, a total of 1.5 kg of ccMA was recovered as a white powder [92].

A *P. putida* KT2440 mutant strain able to perform a sugar-free ccMA production has been also developed by [94]. As also reported previously for the *P. putida* KT2440 strain, the genes encoding the ccMA cycloisomerase (CatB) and the PCA 3,4 dioxygenase (PcaHG) were deleted, and the gene encoding the PCA decarboxylase enzyme (PDC) from *K. pneumoniae* was introduced in the engineered IDPC/pTS110 strain. When 25 mM 4-HBA was supplied as a carbon source, 25 mM vanillic acid was converted into ccMA with a 19.0% yield after 48 h of incubation, evaluated as mol (ccMA)/mol (vanillic acid + 4-HBA) (Table 3): both 4-HBA and vanillic acid were metabolized to PCA, then used to produce biomass and energy via the PCA 3,4-dioxygenase pathway, and ccMA via the PCA decarboxylation pathway ("PCA shunt", Figure 2). The engineered strain generated 0.11 mM ccMA by growing on Japanese cedar lignin pretreated via the alkaline nitrobenzene oxidation process [94].

To bypass the constrain for the application of the *P. putida* KT2440 strain in the ccMA production, and to employ reaction mixtures containing syringyl nucleus compounds as sources for cell growth, the novel *Pseudomonas* sp. NGC7 strain, isolated as syringate-assimilating bacterium [110], was used [93]. The plasmid pTS119 carrying the *pcaHG*, *aroY*, *catA*, and *vanAB* genes was introduced in a NGC7 mutant strain not expressing the PcaHG and CatB enzymes (NGC703/pTS119). In an oxygen-dissolved (DO)-stat fed-batch culture and lowering the DO up to 2.5%, ccMA accumulated up to 35.4 mol% yield (15 g/L) from a mixture of vanillic acid and 4-HBA when the initial feeding rate of the substrates (0.2 mL/h) was increased twice and three times 42 h and 50 h after the inoculation, respectively (Table 3). The engineered strain successfully grew on the sugar cane bagasse alkaline extract prepared via a mild alkaline treatment and accumulated ccMA with a 18.7 mol% yield without sugar feeding (Table 3) [93].

3.2. Bio-Based Plastics Precursors

Besides ccMA, other valuable bio-based plastics precursors can be obtained from the bioconversion of lignin monomers. Moreover, these precursors could be utilized as copolymers to improve the biodegradability of plastic materials [111] or to produce novel biodegradable plastic polymers [112].

Adipic acid is an aliphatic dicarboxylic acid used for the production of various types of plastics, mostly used to produce Nylon 6,6. Due to its many applications, it has a market value of around USD 6 billion, with an annual production of nearly three million tons [113]. Almost all of the adipic acid present on the market is produced from petroleum-based feedstocks, through an energy-intensive process that generates ≈ 10% of anthropogenic N_2O emissions worldwide [114]. Different techno-economic analyses investigated several adipic acid production routes: it was estimated that adipic acid obtained from glucose using a fully biological route would be 20% cheaper (USD 1.36/kg) than the one from chemical route. Another techno-economic analysis demonstrated that using lignin-derived feedstocks instead of glucose as a starting material would reduce the production cost by 50% [115].

To obtain the full biological production of adipic acid from catechol, Kruyer et al. engineered an *E. coli* ΔiscR strain to co-express the catechol 1,2-dioxygenase (CatA) from *Rhodococcus* sp. AN22 and muconic acid reductase (MAR) from *Bacillus coagulans* (Figure 6A) [115]. The engineered strain was cultivated at 37 °C and fed with 1 g/L catechol, carrying out the bioconversion in a two-stage fermentative process: the first 2 h under aerobic conditions and the last 22 h in anaerobic conditions, to have a reducing environment useful to improve ccMA reduction. The bioconversion produced 1.6 mg/L of adipic acid (0.24% yield) from catechol in 24 h (Table 4) [115]. In another work, adipic acid was produced from guaiacol using a BL21(DE3) *E. coli* strain transformed with the pQLinkN plasmid harboring the genes encoding cytochrome P450 GcoAB from *Amycolatopsis* sp. ATCC 39116, catechol 1,2-dioxygenase CatA from *P. putida* and enoate reductase BcER from *B. coagulans* (Figure 6B) [115]. The strain was co-transformed with pGro7 to improve the BcER solubility through chaperonine co-expression. Adipic acid production was performed using resting cells ($OD_{600\,nm}$ = 20) in M9 medium containing 5 mM guaiacol at 37 °C (61% yield in 24 h, Table 4) [114]. Indeed, *P. putida* KT2440 strain was engineered to obtain adipic acid from *p*-coumaric acid and ferulic acid. The gene *pcaF* was knocked-out to funnel the carbon flux towards adipic acid production while the genes encoding 3-ketoadipoyl-CoA reductase PaaH from *E. coli*, 3-hydroxyadipoyl-CoA dehydratase PaaF from *E. coli* and 2,3-dehydroadipoyl-CoA reductase from *Treponema denticola* were integrated into the chromosome under the control of the P_{tac} promoter (Figure 6C) [116]. The fermentation process was optimized by lowering aeration after 24 h to promote the formation of a reducing environment (helpful for adipic acid production) and pulse-feeding glucose to a final concentration of 2.5 g/L: this would allow growth, cofactor recycling and repression of endogenous aromatics metabolism. The fermentation was carried out at 30 °C for 96

h and resulted in 0.76 g/L (18.4% molar yield) of adipic acid from *p*-coumaric acid and ferulic acid (Table 4) [116].

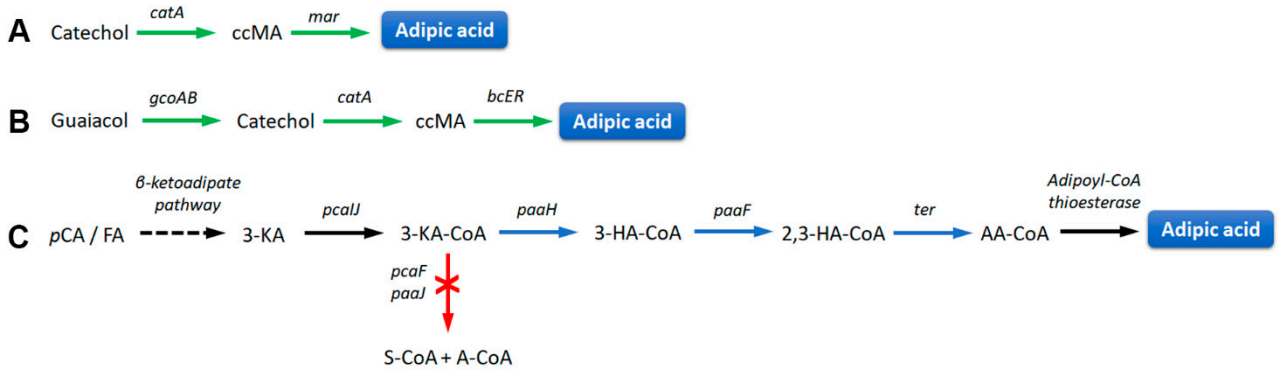

Figure 6. Scheme of adipic acid production from engineered (**A**) *E. coli* ΔiscR [115], (**B**) *E. coli* RARE [114] and (**C**) *P. putida* KT2440 [116]. Encoding genes: *catA*, catechol 1,2-dioxygenase; *mar*, muconic acid reductase; *gcoAB*, cytochrome P450 GcoAB; *bcER*, enoate reductase; *paaH*, 3-ketoadipoyl-CoA reductase; *paaF*, 3-hydroxyadipoyl-CoA dehydratase; *ter*, 2,3-dehydroadipoyl-CoA reductase; pCA, *p*-coumaric acid; 3-KA, 3-ketoadipic acid; 3-KA-CoA, 3-ketoadipoyl-CoA; 3-HA-CoA, 3-hydroxyadipoyl-CoA; 2,3-HA-CoA, 2,3-dehydroadipoyl-CoA; AA-CoA, adipoyl-CoA; S-CoA, succinyl-CoA; A-CoA, acetyl-CoA. Black arrows show the endogenous pathway; green arrows show overexpressed pathways; blue arrows show chromosomal inserted pathways; red crossed arrows identify deleted pathways; dashed arrows show additional multi-step pathways.

Table 4. Metabolic engineering strategies for the production of bio-based plastics and pharmaceuticals precursors.

Product	Host	Starting Material	Deleted Genes	Chromosome Insertion	Overexpressed Genes	Productivity (g/L/h)	Reference
Adipic acid	*E. coli*	Catechol	-	-	*catA, mar*	0.6×10^{-4}	[115]
		Guaiacol	-	-	*gcoAB, catA, bcER*	0.02	[114]
	P. putida KT2440	*p*-Coumarate Ferulic acid	*pcaF*	*paaH, paaF, 2,3-dehydroadipoyil-CoA reductase*	-	0.01	[116]
PHA	*P. putida* KT2440	Ethanol-assisted depolymerized lignin	-	-	*hps, phi, ada*	0.3×10^{-2}	[58]
		p-Coumarate from lignin	*fadAB*	*phaG, alkK, phaC1, phaC2*	-	1.2	[58]
PDC	*P. putida* KT2440	Syringic acid Ferulic acid *p*-Coumarate	*pcaHG, vanAB*	*vanABHR199, ligAB, ligC*	-	0.02	[117]
		p-Coumarate	*pcaHG*	-	*ligAB, ligC*	0.19	[118]
	P. putida PDH	Depolymerized lignin	-	-	*vanAB, ligV, ligAb, ligC, desZ*	0.03	[111]
	N. aromaticivorans DSM12444	Vanillin Vanillic acid	*desC, desD, ligI*	-	-	0.1	[119]
		Depolymerized lignin				0.1×10^{-2}	[119]
2,4-PDCA	*R. jostii* RHA1	Wheat straw Kraft lignin	-	-	*praA*	0.5×10^{-6}	[112]
		Wheat straw Protobind lignin	*pcaHG*	*praA*	*dyp2*	0.007	[120]
2,5-PDCA	*R. jostii* RHA1	Wheat straw	-	-	*ligAB*	0.5×10^{-3}	[112]

Table 4. *Cont.*

Product	Host	Starting Material	Deleted Genes	Chromosome Insertion	Overexpressed Genes	Productivity (g/L/h)	Reference
Gallic acid	*E. coli* MG1655 RARE	*p*-Coumarate	-	-	*hpaBC, fcs, ech, hfd1, pobA*	0.1	[121]
		Ferulic acid	-	-	*fcs, ech, hfd1, vanAB, pobA*	0.1	[121]
	R. opacus PD630	Base-depolymerized AFEX lignin	*protocatechuate 3,4-dioxygenase, putative catechol 2,3-dioxygenase*	*pobA, desV, desA, ligM, metF, ligH*	-	0.6×10^{-2}	[122]
Pyrogallol	*E. coli* DH1	Base-depolymerized Kraft lignin	-	-	*ligM, desA, lpdc*	0.3×10^{-3}	[74]
L-veratrylglycine	*E. coli* BL21(DE3)	Ferulic acid	*metJ*	-	*ejomt, mntN, luxS, metk, AL-11*	0.01	[123]

Polyhydroxyalkanoate (PHA) is a naturally produced biodegradable polymer with promising physical properties [124]. However, extensive biological PHA production is hampered by the cost of the carbon source (i.e., sugars) which accounts for approximately 50% of the process cost. Therefore, using lignin as starting material for PHA production could be an effective strategy to improve the economic and environmental sustainability of the process [58]. Nguien et al. constructed a whole-cell biocatalyst able to convert ethanol-assisted depolymerized lignin into PHA. The *P. putida* KT2440 strain was engineered by introducing the pAWP89 plasmid that allows the expression of hexulose-6-phosphate synthase HPS and 6-phospho-3-hexulose isomerase PHI from *Methylomicrobium alcaliphilum* 20Z, and acetaldehyde dehydrogenase ADA from *Dickeya zeae* under the control of P_{tac} promoter. HPS and PHI allow the use of formaldehyde generated from vanillic acid demethylation by the endogenous enzyme VanAB as a carbon source, and ADA would improve the efficient utilization of ethanol present in the ethanol-assisted depolymerized lignin solution. This strain was cultivated in M9 medium supplemented with depolymerized lignin solution at 30 °C: after 96 h, the fermentation resulted in ≈300 mg/L of PHA (21.3% dcw, Table 4) [58]. Another study utilized a more extensive engineering approach of *P. putida* KT2440 to achieve the conversion of lignin-derived *p*-coumaric acid to PHA. In this latter work, the genes encoding enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase FadB and 3-ketoacyl-CoA thiolase FadA, involved in the PHA degradation pathway, were knocked-out; meanwhile, the genes encoding hydroxyacyl-ACP thiolase PhaG, hydroxyacyl-CoA synthase AlkK and two PHA polymerases PhaC1 and PhaC2 from *P. putida* were integrated into the bacterial genome and overexpressed using the constitutive P_{tac} promoter. The engineered strain was grown in M9 medium supplemented with *p*-coumaric acid at 30 °C and fed with additional *p*-coumaric acid: a titer of ≈950 mg/mL of PHA (≈54% g/g yield) was achieved after 96 h of incubation (Table 4). The same strain produced ≈115 mg/mL of PHA when incubated in modified M9 medium supplemented with 75% sterile soluble lignin stream at 30 °C [125].

2-Pyrone-4,6-dicarboxylic acid (PDC) is a stable chemical intermediate of microbial lignin depolymerization. PDC polyester showed remarkable biodegradability and physical properties [126], making it a promising substitute for TPA in polyester production [118]. A tailored *P. putida* KT2440 strain was engineered to funnel compounds derived from S-, G- and H-lignin monomers (syringic acid, ferulic acid and *p*-coumaric acid, respectively) into PDC production. To achieve this biological funneling, the gene encoding PCA 3,4-dioxygenase PcaHG was knocked-out (to prevent PCA use as a carbon source) and the native *vanAB* gene was substituted with the *HR199 vanAB* from *P. putida* to prevent 3-*O*-methylgallate demethylation while maintaining demethylation activity on syringic acid and PCA. Meanwhile, the genes encoding PCA 4,5-dioxygenase LigAB and CHMS dehydrogenase LigC from *Sphingobium* sp. SYK-6 were integrated into the chromosome under P_{tac} promoter control (Figure 7A) [117]. The fermentation, performed at 30 °C in M9

medium supplemented with 40 mM glucose and 1.5 mM of each lignin monomer, produced 4 mM PDC (93% mol/mol yield) in 36 h (Table 4) [117].

Another study developed and optimized the conversion of *p*-coumaric acid into PDC using an engineered *P. putida* KT2440 strain. Here, the gene encoding PCA 3,4-dioxygenase PcaHG was knocked-out and the genes encoding for PCA 4,5-dioxygenase LigAB and CHMS dehydrogenase LigC from *Sphingobium* sp. SYK-6 were cloned into the pSEVA631 plasmid under the control of P_{tac} promoter (Figure 7B) [118]. Due to the accumulation of 4-HBA in the fermentation broth, the oxygen concentration was raised to 30% to improve the activity of the endogenous enzyme PobA which converts PCA into 4-HBA. The strain was cultivated in M9 medium supplemented with 2.5 g/L glucose and 10 g/L glycerol, and the reaction system was fed with a *p*-coumaric acid solution to maintain a 30 mM concentration. The optimized bioconversion system produced PDC from 120 mM *p*-coumaric acid with a molar yield of ≈100% in 115 h (Table 4) [118].

Quian et al. engineered a *P. putida* PDH strain to convert vanillin and syringaldehyde present in the lignin depolymerization extract [111]. Two different plasmids were built and transferred into the bacterium to improve lignin conversion: (i) pKT230 plasmid harboring the genes encoding vanillate demethylase VanAB from *P. putida* KT2440, vanillin aldehyde dehydrogenase LigV, protocatechuate dioxygenase LigAB and hydroxymuconate-6-semialdehyde dehydrogenase LigC from *Sphingobium* sp. SYK-6; (ii) pJB866 plasmid carrying the gene encoding 3-*O*-methylgallate dioxygenase DesZ from *Sphingomonas paucimobilis* (Figure 7C) [111]. The fermentation in a minimal medium containing 7 g/L glucose and 1.15 mg/mL of Birch extract or Japanese cedar extract at 28 °C produced 0.48 mg/mL and 0.65 mg/mL of PDC, respectively (Table 4).

The *Novosphingobium aromaticivorans* DSM12444 strain was also used to produce PDC from depolymerized lignin, exploiting its innate ability to utilize lignin monomers as a carbon source. The genes *desC*, *desD* and *ligI* were knocked-out from the bacterial genome to funnel lignin monomers metabolism towards PDC production and prevent its degradation [119]. Despite the strain optimization, when cultured in SISnc-V0 media supplemented with 20 mM glucose and a solution of depolymerized lignin containing different lignin monomers at 30 °C, all the aromatic compounds were consumed and 0.5 mM PDC (59% molar yield) was produced only after 77 h. When the engineered strain was cultured in a fed-batch reactor and fed with a solution containing 226 mM vanillic acid, 34 mM vanillin, 550 mM glucose, 15 g/L ammonium sulfate, and 5% (*v*/*v*) DMSO, a total of 26.7 mM (4.9 g/L) PDC was produced in 48 h (Table 4) [119].

Pyridine 2,4-dicarboxylic acid (2,4-PDCA) and **pyridine 2,5-dicarboxylic acid (2,5-PDCA)** are two interesting lignin-derived chemicals that could replace petroleum-derived TPA in polybutyrate adipate terephthalate (PBAT) polymer, thus producing a new plastic polymer [112]. Furthermore, due to soil bacteria's capability to degrade pyridine derivatives [127], the new polymer should be biodegradable. The first attempt to produce these pyridine derivatives was made by engineering *R. jostii* RHA1 to obtain the meta-ring cleavage compound which is then subjected to ammonia cyclization, catalyzed by NH_4Cl, to generate the final product. To produce 2,5-PDCA, the plasmid pTipQ2 harboring the gene encoding protocatechuate 4,5-dioxygenase LigAB from *S. paucimobilis* was transferred into *R. jostii* RHA1; meanwhile, to produce 2,4-PDCA, the plasmid pTipQ2 carrying the gene encoding protocatechuate 2,3-dioxygenase PraA from *Paenibacillus* sp. JJ-1b was employed [112]. The engineered strains were cultivated in M9 medium (containing NH_4Cl) supplemented with either 1% wheat straw or 0.5% Kraft lignin at 30 °C. The fermentation of LigAB-expressing strain produced 125 mg/L of 2,4-PDCA from wheat straw (after 9 days) and 53 mg/L of 2,4-PDCA from Kraft lignin (after 4 days) (Table 4). The fermentation of PraA-expressing strain produced 106 mg/L of 2,5-PDCA from wheat straw (after 9 days) while no product formation was observed when fed with Kraft lignin (Table 4) [112]. The same research group optimized the 2,4-PDCA production through further strain engineering: in detail, the gene encoding LigAB was integrated into the chromosome under control of the engineered promoter P_{tpc5}, the gene *pcaHG* was knocked-out to block the

competing β-ketoadipate pathway and the strain was transformed with the expression plasmid pTipQC2 harboring the genes encoding peroxidase Dyp2 from *Amycolatopsis* sp. 75iv2, to improve lignin oxidation rate [120]. The improved whole-cell biocatalyst was cultivated in M9 medium (containing NH_4Cl) supplemented with either 1% wheat straw or 1% Green Value Protobind lignin at 30 °C. The fermentation resulted in 330 mg/L of 2,4-PDCA produced from wheat straw and 240 mg/L of 2,4-PDCA produced from Green Value Protobind lignin in 40 h (Table 4) [120].

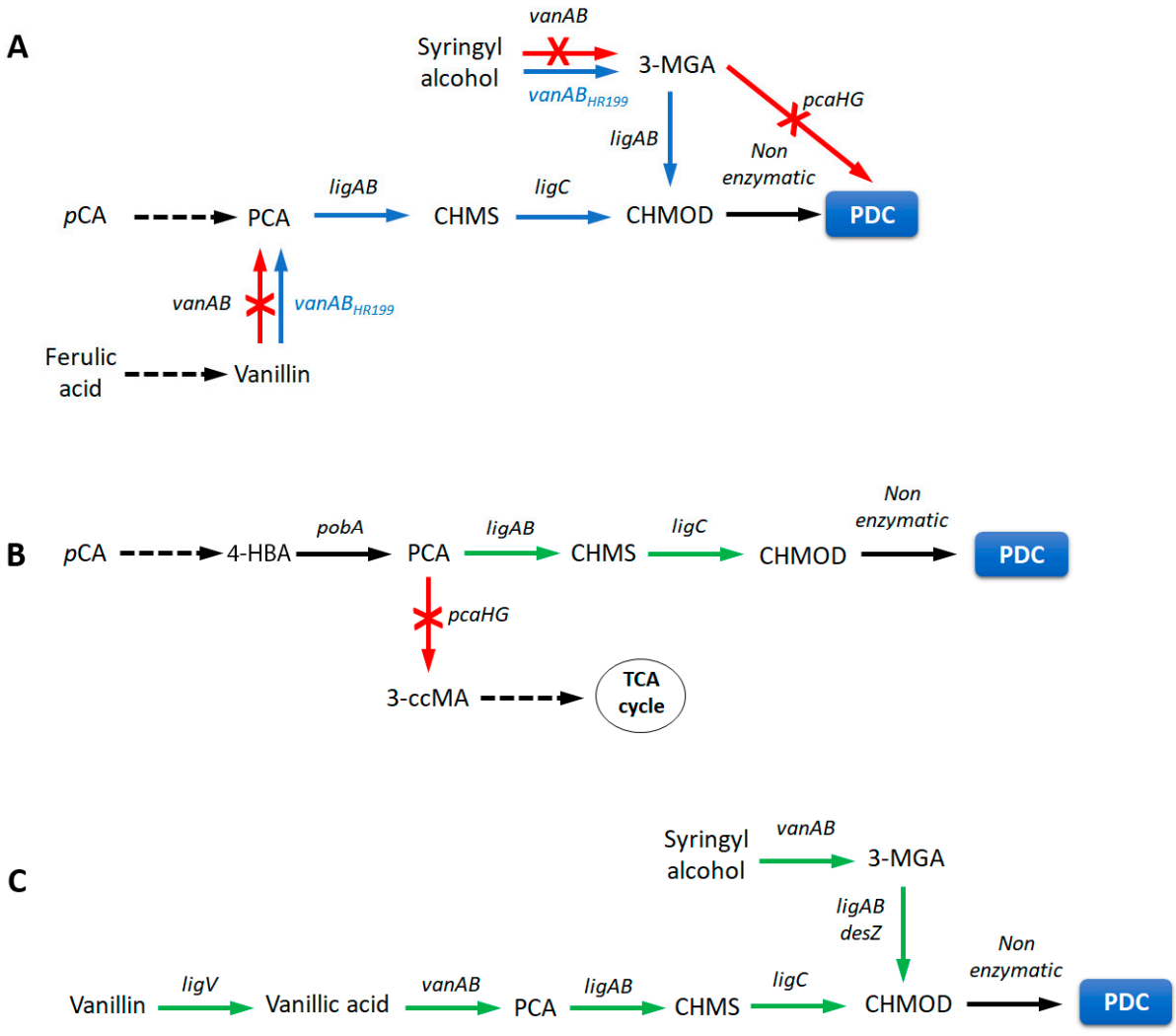

Figure 7. Scheme of PDC production from engineered (**A**,**B**) *P. putida* KT2440 [117,118] and (**C**) *P. putida* PDH [111]. Encoding genes: *pcaHG*, PCA 3,4-dioxygenase; *vanAB*, vanillate demethylase; *ligAB*, PCA 4,5-dioxygenase; *ligC*, CHMS dehydrogenase; *pobA*, *p*-hydroxybenzoate hydroxylase; *ligV*, vanillin dehydrogenase; *desZ*, 3-*O*-methylgallate dioxygenase; pCA, *p*-coumaric acid; PCA, protocatechuic acid; CHMS, 4-carboxy-2-hydroxy-cis,cis-muconate 6-semialdehyde; CHMOD, 4-carboxy-2-hydroxy-6-methyoxy-6-oxohexa-2,4-dienoate; 3-MGA, 3-methoxygallate; 4-HBA, 4-hydroxybenzoic acid; 3ccMA, 3-carboxy-ccMA. Black arrows show the endogenous pathway; green arrows show overexpressed pathways; blue arrows show chromosomal inserted pathways; red crossed arrows identify deleted pathways; dashed arrows show additional multi-step pathways.

3.3. Pharmaceuticals

Interestingly, lignin could also represent an interesting source of bioactive molecules and pharmaceutical precursors [64]. The development of biotechnological processes to produce these high-value compounds could represent the driving force to establish competitive lignin biorefineries [128].

Gallic acid is a natural phenolic compound occurring in many different plant species and showing several interesting biological activities, such as anti-cancer, anti-oxidant and anti-inflammatory effects among others [129]. In addition, this compound is utilized in the food and cosmetic industries as preservative, and is a precursor for the production of various value-added products [130]. Nowadays, gallic acid is mainly obtained from chemical or enzymatic hydrolysis of tannins, making its production a high-cost process dependent on raw material quality and availability [122]. Fu et al. developed two whole-cell biocatalysts to produce gallic acid lignin-derived aromatics from *p*-coumaric acid and from ferulic acid [121]. The strain of choice for this bioconversion was *E. coli* MG1655 RARE [103]. To construct the biocatalyst using *p*-coumaric acid as substrate, three different expression plasmids were introduced in *E. coli*: pETDuet-1 harboring genes encoding feruloyl-CoA synthetase FCS and enoyl-CoA hydratase/aldolase ECH from *P. putida*, pRSFDuet-1 carrying genes encoding aldehyde dehydrogenase HFD1 from *S. cerevisiae* and pACYCDuet-1 plasmid to express vanillic acid *O*-demethylase VanAB from *P. putida* and the *p*-hydroxybenzoate hydroxylase PobA-Y385F variant from *P. putida* (Figure 8A, top) [121]. The biocatalyst to convert ferulic acid into gallic acid was built using the same plasmids, but with the plasmid pACYCDuet-1 carrying the genes encoding the two-component flavin-dependent monooxygenase HpaBC from *E. coli* instead of *vanAB* gene (Figure 8B, bottom). The bioconversion was performed with resting cells in 200 mM phosphate buffer at pH 8.0 and 30 °C, at 10 mM initial substrate concentration and feeding with additional 10 mM of substrate after 6 h. Starting from *p*-coumaric acid, ≈19.5 mM gallic acid (≈98% yield) was produced in 36 h; meanwhile, using ferulic acid, ≈19.9 mM gallic acid (≈99% yield) was produced in 36 h (Table 4) [121]. Gallic acid was also generated from base-depolymerized lignin using an engineered *R. opacus* PD630 strain, capable of utilizing S-, G- and H-lignin monomers as substrates. The Y385F and T294A substitutions were introduced in the endogenous enzyme PobA to improve the hydroxylation activity, and the genes encoding protocatechuate 3,4-dioxygenase and a putative catechol 2,3-dioxygenase were deleted to block gallate and PCA biodegradation pathways. Then, to improve S-lignin monomers utilization, the genes encoding aldehyde hydrogenase DesV, THF-dependent *O*-demethylases DesA and LigM, and the tetrahydrofolate recycling system MetF and LigH from *Sphingobium* sp. SYK-6 were integrated in the genome (Figure 8B) [122]. The engineered strain was cultivated in M9 medium supplemented with 10 mM glucose and 0.5 g/L of soluble base-depolymerized AFEX lignin as substrate at 30 °C: after 48 h of incubation, 1.8 mM gallic acid was produced (Table 4) [122].

Pyrogallol is a phenolic compound used as a substrate for the chemical synthesis of different biologically active molecules [131] that could have potential use as anti-proliferative agent on cancer cells [74]. Pyrogallol is produced through the thermal decarboxylation of gallic acid [131]: accordingly, its current industrial production shows the same drawbacks as the gallic acid one. Wu et al. attempted the production of pyrogallol from syringic acid obtained from base-catalyzed oxidation of Kraft lignin using an engineered *E. coli* DH1 strain [74]. The biochemical pathway was constructed by inserting into the pBbE1a plasmid the genes encoding the enzymes tetrahydrofolate-dependent *O*-demethylases LigM and DesA from *Sphingomonas* sp. SYK-6, and the decarboxylase Lpdc from *Lactobacillus plantarum* WCFS1 (Figure 8C) [74]. The bioconversion of lignin-derived syringate to pyrogallol was assayed using both growing cells and resting cells. The fermentation was performed in LB medium supplemented with 20 g/L glucose and 1 g/L of lignin-derived syringate at 37 °C and produced 7.3 mg/L pyrogallol and 18 mg/L of gallic acid. The bioconversion using resting cells was carried out in M9 medium supplemented with 10 g/L glucose and 0.5 g/L of lignin-derived syringate at 30 °C: this approach improved the gallate production (59.6 mg/L) but the pyrogallol production was similar to the fermentation system (6.2 mg/mL) (Table 4) [74].

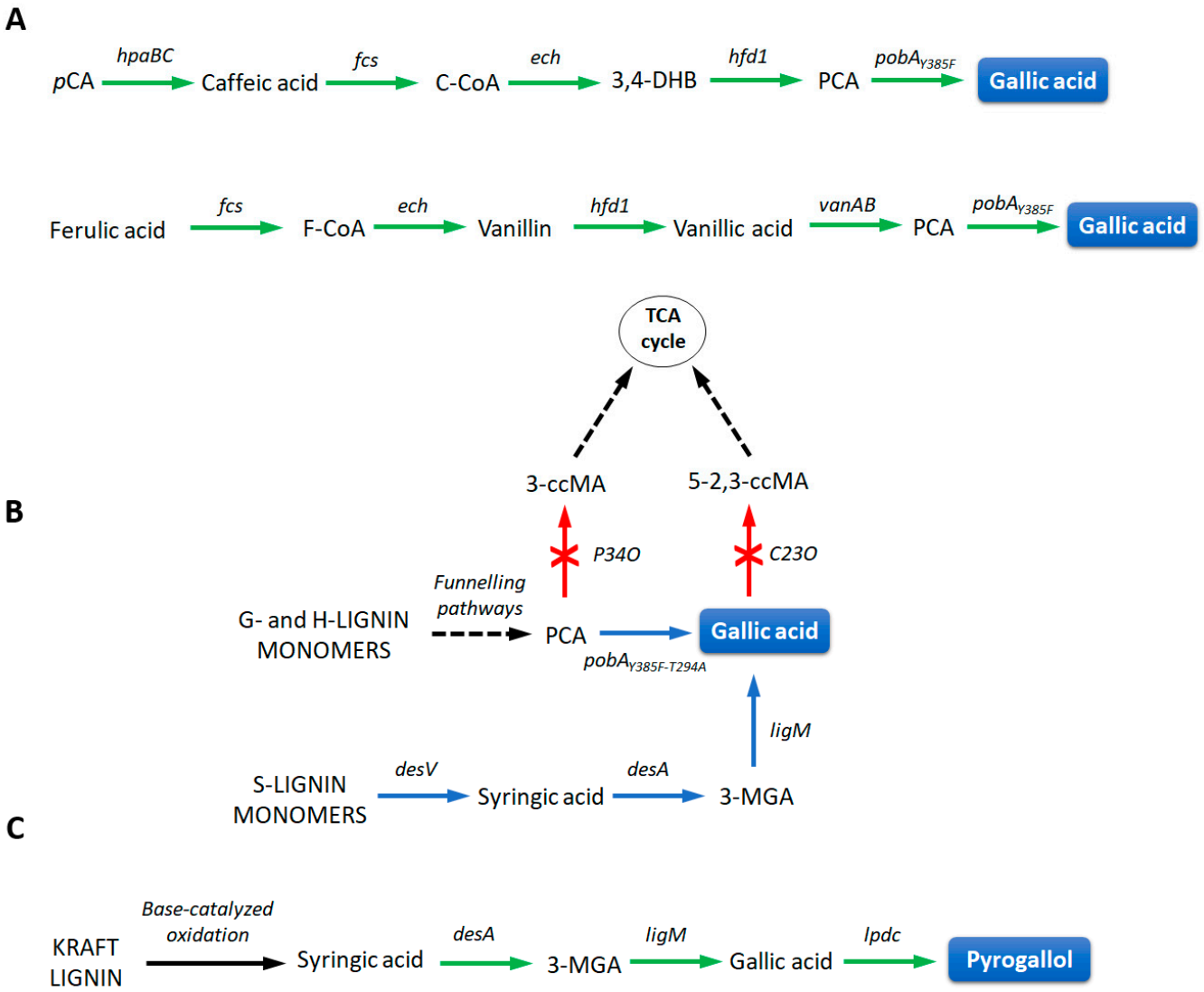

Figure 8. Scheme of gallic acid production from (**A**) engineered *E. coli* RARE [121] and (**B**) *R. opacus* PD630 [122], and of pyrogallol production from (**C**) engineered *E. coli* DH1 [74]. Encoding genes: *hpaBC*, two-component flavin-dependent monooxygenase; *fcs*, feruloyl-CoA synthetase; *ech*, enoyl-CoA hydratase/aldolase; *hfd1*, aldehyde dehydrogenase; *pobA*, *p*-hydroxybenzoate hydroxylase; *vanAB*, vanillate demethylase; *desV*, aldehyde hydrogenase; *desA* and *ligM*, THF-dependent *O*-demethylases; *P34O*, protocatechuate 3,4-dioxygenase; *P23O*, putative catechol 2,3-dioxygenase; *lpdc*, decarboxylase. pCA, *p*-coumaric acid; C-CoA, caffeoyl-CoA; 3,4-DHB, 3,4-dihydroxybenzoic acid; F-CoA, feruoyl-CoA; 5-2,3-ccMA, 5-carboxy-2,3-dihydroxymuconate 6-semialdehyde; 3ccMA, 3-carboxy-ccMA; 3-MGA, 3-methoxygallate. Black arrows show the endogenous pathway; green arrows show overexpressed pathways; blue arrows show chromosomal inserted pathways; red crossed arrows identify deleted pathways; dashed arrows show additional multi-step pathways.

3,4-Dihydroxyphenyl-L-alanine (L-DOPA) is the precursor of dopamine and one of the most effective drugs used to treat Parkinson's disease. Due to its role, the global market demand is approximately 250 tons per year with a market value of approximately USD 101 billion [132]. Currently, L-DOPA is produced via chemical synthesis and extraction from plants; however, these production methods are not sustainable [133]. Galman et al. developed a two-stage one-pot process to produce biobased L-veratrylglycine, a precursor of L-DOPA [123]. In the first step, ferulic acid is methylated to 3,4-dimethoxycinnamic acid: the plasmid pET28 harboring the gene encoding a variant of *O*-methyltransferase EjOMT enzyme (I133S/L138V/L342V) from *Eisenia japonica* and the plasmid p-ACYCDuet-1 carry-

ing the gene encoding S-adenosylhomocysteine nucleosidase MntN, S-ribosylhomocysteine lyase LuxS and a variant of SAM synthetase MetK enzyme (I303V) from *E. coli* were transferred into the *E. coli* BL21(DE3) strain. To increase the methionine availability, the endogenous transcriptional repressor MetJ was deleted from the E. coli's genome. The engineered strain (at 0.7% *w*/*v*), when incubated in M9 medium supplemented with 5 mM D,L-methionine and 3 mM ferulic acid at 30 °C, produced 0.6 g/L of 3,4-dimethoxycinnamic acid (99% conversion) in 48 h. In the second step, an engineered *E. coli* BL21(DE3) strain overexpressing a variant of the ammonia lyase AL-11 enzyme (Q84V) was added to the reaction mixture (0.8% *w*/*v*) together with 4 M ammonium carbamate: after an additional 18 h of incubation at 30 °C, the complete conversion in L-veratrylglycine into L-DOPA (>99% ee) was obtained (Table 4) [123].

4. Perspectives

The cost-effectiveness of biological lignin valorization largely depends on converting the lignin-derived aromatics to value-added compounds. To improve the economic viability of the overall lignin valorization process, especially at the industrial scale, and to be cost-competitive with petroleum routes of chemical synthesis, several concepts/tools/strategies seem particularly relevant to fill the gap:

- the screening/use of engineered promoters with varying strength that could lead to tunable systems aimed at maximization of protein expression of selected enzymes of the relevant pathways;
- the use of metabolic engineering and protein engineering approaches to remove any bottleneck of the relevant pathways;
- the optimization of fermentation conditions;
- the efficient supply of large quantities of lignin in a relatively uniform, purified and biocompatible form;
- the optimization of lignin depolymerization to yield a substrate with high monomer concentrations;
- the limitation of toxicity of lignin degradation products;
- the optimization of the products' separation process that has been estimated to account for over 60% of production costs [134].

These points are expected to guide future research in the field.

5. Conclusions

The future economic success of lignocellulosic-based biorefineries is tied to the valorization of lignin. Lignin is an excellent material for producing a number of value-added bioproducts. Microorganisms and enzymes are well suited to handle the inherent recalcitrant properties of lignin, its heterogeneity and its toxicity. As illustrated, metabolic engineering (also supported by protein engineering to optimize selected enzymatic steps) resulted in microbial cell factories for the production of relevant chemicals from lignin at the laboratory scale and at the pilot scale.

Biological lignin valorization has not yet been conducted at an industrial/commercial scale, so techno-economic analyses have not been fully evaluated. A recent study highlighted that the biological conversion of lignin into bioplastics had significant potential to improve the profitability of lignin valorization [135]. A "plug-in processes of lignin' with the integration of five fractionation pretreatments has been set up: the optimized process could enable a minimum polyhydroxyalkanoate selling price at as low as USD 6.18/kg, with operating costs accounting for about 47–58% of total production [135]. Overall, low costs of lignin fractionation and of downstream separation processes are essential to the economic success of lignin valorization. The development of innovative approaches, such as synthetic biology and metabolic engineering, could be helpful to increase the production yields and to design new bioconversion pathways thus reducing the price of the final products and further increasing and diversifying the portfolio of products from lignin.

Although at present the bacterial lignin valorization is still a "field of dreams" [64], growing research and innovation will make it a reality in the short period and a central pillar in sustainability.

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