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Guiding stars to the field of dreams: Metabolically engineered pathways and microbial platforms for a sustainable lignin-based industry

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Dedicated to Dr. Judith Becker (* 1.2. 1981, † 27.4.2021), a gifted metabolic engineer of sustainable cell factories and our cherished colleague and friend at the Institute of Systems Biotechnology of Saarland University

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Catechol
Protocatechuate
Muconate

ABSTRACT

Lignin is an important structural component of terrestrial plants and is readily generated during biomass fractionation in lignocellulose processing facilities. Due to lacking alternatives the majority of technical lignins is industrially simply burned into heat and energy. However, considering its vast abundance and a chemically interesting richness in aromatics, lignin is presently regarded both as the most under-utilized and promising feedstock for value-added applications. Notably, microbes have evolved powerful enzymes and pathways that break down lignin and metabolize its various aromatic components. This natural pathway atlas meanwhile serves as a guiding star for metabolic engineers to breed designed cell factories and efficiently upgrade this global waste stream. The metabolism of aromatic compounds, in combination with success stories from systems metabolic engineering, as reviewed here, promises a sustainable product portfolio from lignin, comprising bulk and specialty chemicals, biomaterials, and fuels.

1. Introduction

Lignin, famous and notorious alike, has already occupied scientists for several decades (Adler, 1957; Brauns and Hibbert, 1933; Nimz, 1974; Ritter, 1925), and the worldwide fascination surrounding this biopolymer is still unbroken, if not rising constantly. Lignin is a major component of lignocellulosic biomass, besides cellulose and hemicellulose (Abdelaziz et al., 2016), and is considered to be the second most abundant plant biopolymer on Earth (Boudet, 1998). Massive amounts of technical lignins are generated as side stream during biomass fractionation in lignocellulose-processing industrial branches all around the world (Bruijnincx et al., 2015).

The pulp and paper industry accumulates approximately 50 million metric tons of lignin every year in the course of 130 million metric tons of kraft pulp produced (Bruijnincx et al., 2015). Hereby, the intrinsic heterogeneity and recalcitrance of the technical lignin streams heavily impair their further utilization (Beckham et al., 2016; Rinaldi et al., 2016). In consequence, the majority of this waste is simply burned to generate heat and power (De Wild et al., 2014), whereas non-energetic applications are restricted to selected niches, e.g., vanillin production (Abdelaziz et al., 2016). Even more significant are second-generation biorefineries (Beckham et al., 2016), which generate up to 1.5 kg lignin per liter of produced cellulosic ethanol (Bruijnincx et al., 2015). Considering the use of the six major agricultural residues (corn stover, rice straw, sugarcane straw/bagasse, wheat straw, barley straw, soybean hulls) projects 198 million metric tons of produced lignin, giving a glimpse at the global opportunities for lignin upgrading (Gonçalves et al., 2020). Notably, lignocellulosic biorefineries, including bioethanol plants, are threatened in their economic viability and sustainability, if lignin's major utilization is restricted to low-value energetic purposes

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(Beckham et al., 2016; Rinaldi et al., 2016). These issues urgently require innovative approaches, to make the leap from a generally neglected waste material into an acknowledged source of value-added products (De Wild et al., 2014).

The major hope and enormous potential of lignin, actually lies within the origin of its "flaws", namely its unique aromaticity (Bruijnincx et al., 2015). Lignin is the only renewable source of aromatic compounds (Tuck et al., 2012), and thus, holds within the tempting opportunity to escape our strong dependency on petroleum-derived aromatics (De Wild et al., 2014; Rinaldi et al., 2016). To make lignin accessible for such value-added applications, the polymer has to be depolymerized, whereby, bio-based processes for lignin-valorization gain increasingly attention to overcome the problems arising from the heterogeneity of the depolymerized raw material (Abdelaziz et al., 2016; Linger et al., 2014).

Naturally, a community of different microorganisms is involved in lignin degradation and assimilation (Brink et al., 2019). Prominently, the bacterial metabolic networks that mediate the degradation of the involved various aromatics are regarded as door openers to integrate lignin into a biotechnological valorization chain (Abdelaziz et al., 2016; Becker and Wittmann, 2019; Beckham et al., 2016; Li and Zheng, 2020). Several success stories have recently demonstrated the extraordinary potential of systems metabolically engineered bacteria to convert lignin waste streams into an ample product portfolio that comprises chemicals, materials, fuels, and flavors (Becker and Wittmann, 2019).

In this review, we outline the necessary strategies and technologies from lignin to value-added products using microbial cell factories. Because they display the treasure maps and blueprints for the metabolic engineer, we cover the pathways for approximately fifty aromatics of relevance, a complexity that has been assembled from various studies over the past decades and is continuously renewed and extended. We further showcase powerful strategies of systems metabolic engineering to upgrade microbes for aromatics conversion and highlight seminal success stories for lignin valorization.

2. Two sides of the same coin: lignins in nature and in industry

2.1. Natural lignin

Forestry, fast growing perennial grasses, and agro-industrial residues are the major depots of lignocellulosic biomass (Gonçalves et al., 2020; Van den Bosch et al., 2018) (Fig. 1). Structurally, lignin is tightly embedded in the complex architecture of the plant biomass and makes up 15–30% of its dry weight (Bugg et al., 2011a) to reinforce plant cell walls, bestow steadiness and protection, and ease water transport (Ralph et al., 2004). Notably, lignin biopolymers exhibit a pronounced heterogeneity and intricacy at several levels (del Río et al., 2020; Rinaldi et al., 2016), whereby lignin's composition differs between plants (Boerjan et al., 2003), within the same plant (Abdelaziz et al., 2016) and is subjected to multiple environmental factors (Boudet, 1998; Rinaldi et al., 2016).

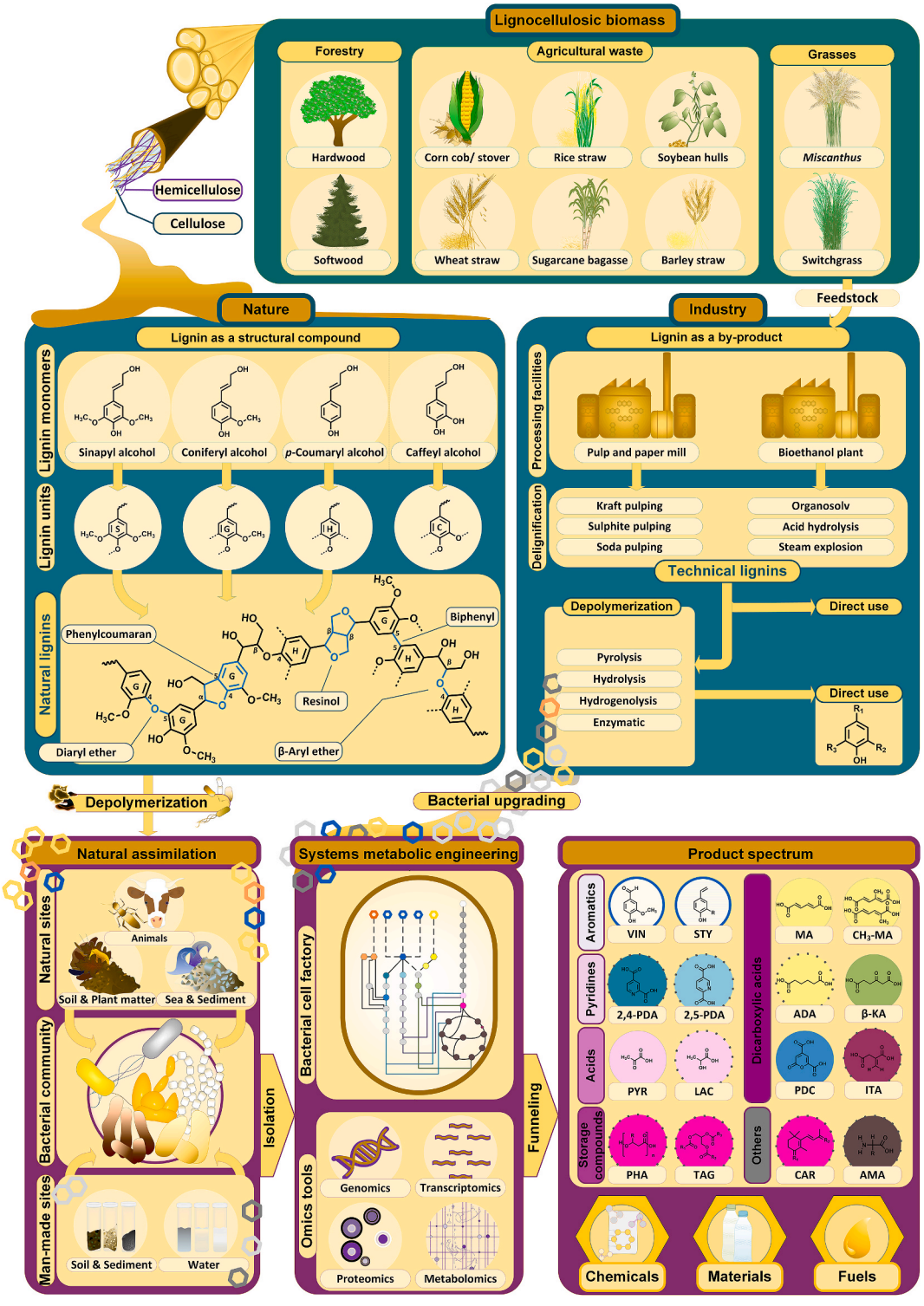
Chemically, lignin majorly consists of three differently methoxylated hydroxycinnamyl alcohols (monolignols), namely coniferyl (G)-, *p*-coumaryl (H)-, and sinapyl (S)-alcohol (Boerjan et al., 2003; Ralph et al., 2004) (Fig. 1). These archetypical building blocks are generated in the plant phenylpropanoid pathway (Rinaldi et al., 2016). However, novel monomer-variants are constantly discovered (Boudet, 1998; del Río et al., 2020; Ralph et al., 2004), underlining lignin's enormous chemical plasticity. The assembly of lignin polymers occurs by oxidative coupling of radical intermediates, which are enzymatically generated from the building blocks by peroxidases and laccases (Rinaldi et al., 2016). Miscellaneous chemical functionalities, as well as mesomeric effects of the radicals, give rise to a variety of structural units, whereby the prevalence of certain bond types is a direct consequence of the monomeric composition (Ralph et al., 2004) (Fig. 1). Besides other more resistant carbon-carbon and carbon-ether bonds, the β-*O*-4 (or β-aryl)-ether displays the most abundant linkage type in native lignins (45–84%) (Rinaldi et al., 2016), which was also suggested to be of high importance for further valorization (Lancefield et al., 2016). Particularly interesting, C-lignin, solely composed of caffeyl (C)-alcohol monolignols (Chen et al., 2013) has a curiously linear and homogenous structure, setting it apart from "common" lignins, likewise fueling intensive efforts to understand its unique properties for potential usage in lignin valorization (Berstis et al., 2016).

2.2. Technical lignin

Both in archetypical (pulp and paper mills) and emerging (biorefineries) lignocellulose fractionation facilities, copious amounts of technical lignins are generated as a by-product (Bruijnincx et al., 2015). The cooking process for solubilization and separation of lignin from the carbohydrate fraction can proceed by different methods (Fig. 1), as excellently reviewed by (Abdelaziz et al., 2016; Rinaldi et al., 2016; Schutyser et al., 2018; Van den Bosch et al., 2018). Lignin is traditionally neglected in the industrial valorization of lignocellulosic biomass, so that most fractionation technologies are tailored to provide high quality cellulosic streams, simultaneously taking into account drastical changes in the structure of lignin (Rinaldi et al., 2016; Van den Bosch et al., 2018). The residual technical lignins not only differ depending on the fractionation method, but can also contain unconverted carbohydrates, minerals, and process chemicals, disadvantageous for value-added applications (De Wild et al., 2014). Thus, the choice of the fractionation method has severe influence on the further opportunities for lignin valorization (De Wild et al., 2014; Schutyser et al., 2018).

Among the available alkaline fractionation methods (Kraft, sulphite, and soda pulping) in pulp and paper mills, the most common and industrially relevant is Kraft pulping (Abdelaziz et al., 2016; Rinaldi et al., 2016; Van den Bosch et al., 2018), setting free approximately 50 million tons of lignin (Bruijnincx et al., 2015). Here, lignocellulose, most prominently softwood, is cooked at high temperatures of 155–175 °C in an aqueous solution of NaOH and Na_2S (white liquor), where ether bond cleavage is achieved by OH^- and HS^- ions (Abdelaziz et al., 2016). The process generates next to the actual product in form of solid cellulose-enriched pulp, also a liquid fraction containing soluble lignin and hemicellulose constituents (black liquor), whereby the Kraft lignin is isolated from the alkaline black liquor *via* neutralization (Van den Bosch et al., 2018). Besides a low sulphur content (1–3%) (Van den Bosch et al., 2018), Kraft lignin is prominently characterized by a highly condensed, cross-linked structure, caused by an exchange of the lignin-typical β-*O*-4 ether by C–C bonds, aggravating further upgrading for commercial purposes (Rinaldi et al., 2016; Van den Bosch et al., 2018). Thus, the high volumes of Kraft lignin streams are largely burned for the generation of heat and power, and are used for Na_2S regeneration (Van den Bosch et al., 2018).

Complementary, in biorefineries other biomass fractionation methods appear to be of higher relevance (e.g., organosolv, acid-hydrolysis, steam explosion) (De Wild et al., 2014) (Fig. 1), and are under constant development to ultimately enable a valorization of all lignocellulosic components, that means also of hemicellulose and lignin (Galbe and Wallberg, 2019). The organosolv process is regarded as especially promising for value-added applications of lignin (Borand and Karaosmanoğlu, 2018). Here, delignification is based on different solvent/water mixtures, whereby the biomass is cooked at temperatures of 180–195 °C causing a high removal of lignin along with small amounts of cellulose (Rinaldi et al., 2016). Favorably, certain organosolv lignins contain higher fractions of the readily cleavable β-*O*-4 linkages (Rinaldi et al., 2016; Schutyser et al., 2018), and are featured by a stable quality (Abdelaziz et al., 2016), as well as purity (Borand and Karaosmanoğlu, 2018; De Wild et al., 2014). However, based on lacking economic competitiveness with other biomass treatment methods, the organosolv process so-far only played a minor role for industrial application (Borand and Karaosmanoğlu, 2018; Rinaldi et al., 2016).



(caption on next page)

Fig. 1. A field of dreams: From lignin-derived aromatics to value-added products by harnessing metabolically engineered bacteria. Naturally, lignin, next to cellulose and hemicellulose, is an important structural component of terrestrial plant biomass (Becker and Wittmann, 2019). Lignin is composed of different aromatic building blocks (Chen et al., 2013; del Río et al., 2020; Ralph et al., 2004), whereby radical polymerization gives rise to variable bonding motifs (blue) (Rinaldi et al., 2016). Notably, high amounts of technical lignin are generated as a by-product in pulp and paper mills or bioethanol plants *via* different biomass fractionation methods (Abdelaziz et al., 2016; De Wild et al., 2014), with forestry, agricultural residues and grasses as the main lignocellulose resources (Gonçalves et al., 2020; Van den Bosch et al., 2018). Next to depolymerizing lignin into aromatic compounds (Abdelaziz et al., 2016; Van den Bosch et al., 2018), a promising opportunity for its valorization, relies on the natural arsenal of microorganisms, capable of using natural lignin-derived aromatics as carbon and energy source (Beckham et al., 2016). Interesting bacterial candidates, stemming from microbial communities, may either be isolated from natural or man-made environments rich in aromatics (Brink et al., 2019), including soils (Margesin et al., 2021; Seto et al., 1995), plant matter (Shi et al., 2013), sea water (González et al., 1997; Numata and Morisaki, 2015), marine sediments (Ohta et al., 2012), or animals (Degrassi et al., 1995; Zhou et al., 2017). After a profound characterization *via* systems metabolic engineering tools (Chae et al., 2017), isolates may find application as cell factories for an ample product portfolio (Becker and Wittmann, 2019). Various works have shown the suitability of aromatics to serve as a substrate for the bacterial production of: adipate (ADA) (Niu et al., 2020), amino acids (AMA) (Lee et al., 2010), carotenoids (CAR) (Chen and Wan, 2021), as suggested by (Takaichi et al., 1990), itaconate (ITA) (Elmore et al., 2021), β-ketoadipate (β-KA) (Okamura-Abe et al., 2016), lactate (LAC) (Johnson and Beckham, 2015), *cis, cis*-muconate (MA) (Vardon et al., 2015), methylated *cis, cis*-muconate-variants (CH_3-MA) (Kohlstedt et al., 2018), polyhydroxyalkanoates (PHA) (Borrero-de Acuña et al., 2020), pyridine-2,4-dicarboxylate (2,4-PDA), pyridine-2,5-dicarboxylate (2,5-PDA) (Mycroft et al., 2015), 2-pyrone-4,6-dicarboxylate (PDC) (Otsuka et al., 2006), pyruvate (PYR) (Johnson and Beckham, 2015), vanillin (VIN) (Fleige et al., 2016), styrene derivatives (STY) (Williamson et al., 2020), triacylglycerols (TAG) (Amara et al., 2016). The product colors refer to the metabolic scheme of the bacterial cell factory, with products directly obtained from aromatic metabolism depicted in plain colors, and products requiring further conversion with a dotted line. Parts of the figure were adapted from previous works (Abdelaziz et al., 2016; Becker and Wittmann, 2019; Ralph et al., 2004).

2.3. Depolymerization of lignin – the key towards value-added applications by bacterial cell factories

Next to a direct, material application of technical lignins at low value (e.g., adhesives) (Abdelaziz et al., 2016; Rinaldi et al., 2016), higher-value opportunities arise from their depolymerization into aromatic chemicals (Van den Bosch et al., 2018) (Fig. 1). Most lignin depolymerization strategies cause variable streams of low-molecular weight oligo- and monomeric aromatics that are difficult to separate (Abdelaziz et al., 2016; Schutyser et al., 2018), which in terms of monomer yield, and selectivity, appears limiting for a direct production of chemicals from lignin (Beckham et al., 2016; Van den Bosch et al., 2018). This is one reason, why microbes, that also naturally face aromatic substrate mixtures, appear suitable for an upgrading of lignin waste streams (Linger et al., 2014).

Also, for microbial valorization, the depolymerization of lignin into low molecular weight compounds is crucial to ensure its bioavailability for further metabolic upgrading (Abdelaziz et al., 2016; Becker and Wittmann, 2019). However, the different aromatics obtained from different lignin depolymerization approaches and lignin types (Abdelaziz et al., 2016; Lancefield et al., 2016; Schutyser et al., 2018), may not always be covered by the natural substrate spectrum of a single microbe, giving valuable hints for metabolic engineering approaches (see **6.2**). Undoubtedly, tailoring depolymerization strategies to yield a product spectrum, which is suitable for subsequent biological upgrading is an important pillar in microbial lignin valorization (Beckham et al., 2016). This is also underpinned by a couple of studies aiming at high yields of selected aromatics for an optimal subsequent metabolism by microbes (Barton et al., 2018; Rodriguez et al., 2017; van Duuren et al., 2020).

2.3.1. Biobased depolymerization of lignin

Naturally, the depolymerization of the lignin fraction of lignocellulose into low molecular weight aromatics is achieved by microorganisms (Fig. 1), and the associated enzyme multiplicity emerges as an alternative to chemical depolymerization methods (Bugg et al., 2020; Cagide and Castro-Sowinski, 2020; Picart et al., 2015; Pollegioni et al., 2015). Especially, white-rot fungi (see **3.2**) are well-known for an ample repertoire of oxidative lignin-degrading enzymes (laccases, different heme-containing peroxidases: e.g., lignin peroxidases, manganese-dependent peroxidases, versatile peroxidases, dye-decolorizing peroxidases), and several accessory enzymes (e.g., glyoxal oxidase) supporting lignin degradation (Janusz et al., 2017). In the past few years, also bacteria (see **3.1**) shifted into the academic focus (Ahmad et al., 2010; Salvachúa et al., 2015), and genomic analysis suggested that lignin-degrading bacteria are characterized by variable lignin oxidation mechanisms in terms of enzyme variability (Granja-Travez et al., 2020). As an example, dye-decolorizing peroxidases were characterized detailly for the two soil-bacteria *Amycolatopsis* sp. strain ATCC 39116 (Brown et al., 2012) and *Rhodococcus jostii* RHA1 (Ahmad et al., 2011; Vignali et al., 2018), and also, multi-copper oxidases were reported to be involved in lignin oxidation by *Pseudomonas* strains (Granja-Travez and Bugg, 2018) (Table 1).

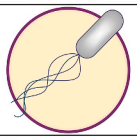
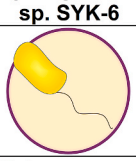
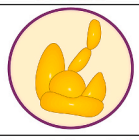
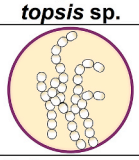
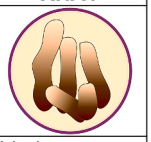
Currently, knowledge on the exact mechanisms underlying the biotransformation of polymeric lignin is still incomplete (Bugg et al., 2020). The β-*O*-4-bond, which is prominently found in natural lignins of different biomass types (Rinaldi et al., 2016), is suspected to serve as the major attack point for enzymatic lignin depolymerization, providing different possibilities for oxidative cleavage or oxidation (Bugg et al., 2020). For instance, vanillin was detected after incubation of the lignin peroxidase DypB from *R. jostii* RHA1 with a β-aryl ether model compound, hinting at a Cα-Cβ-cleavage mechanism (Ahmad et al., 2011), which is also suggested for fungal lignin peroxidases and other bacterial dye-decolorizing peroxidases (Bugg et al., 2020). This cleavage mechanism might be the origin of the commonly observed aromatic product spectrum obtained from the microbial depolymerization of lignocellulosics, lignin or lignin model oligomers (Bugg et al., 2011a), comprising differently substituted aldehydes (e.g., vanillin, syringaldehyde) or benzoates (e.g., vanillate) (Lancefield et al., 2016; Mathews et al., 2016; Ramachandra et al., 1988; Sainsbury et al., 2013).

Additionally, apart from oxidation of the β-*O*-4-bond, another group of interesting enzymes are β-etherases involved in reductive β-aryl ether cleavage, relying on glutathione as a co-factor (Bugg et al., 2020; Picart et al., 2015). The respective pathway was investigated detailly in *Sphingobium* sp. strain SYK-6 (Higuchi et al., 2020; Masai et al., 2007a; Meux et al., 2012), which is well known for its impressive range of metabolic pathways for the degradation of other lignin-associated biaryls (Kamimura et al., 2017b), and, also, in *Novosphingobium aromaticivorans* DSM 12444 (Kontur et al., 2018). Mostly focusing on the dimeric lignin model compound guaiacylglycerol-β-guaiacyl ether, whereby for instance the release of guaiacol is observed (Higuchi et al., 2020; Kontur et al., 2018; Masai et al., 2007a), further studies indicated, that β-etherases might also be involved in the depolymerization of real lignin (Bugg et al., 2020; Picart et al., 2015).

Even though, the application of ligninolytic enzymes for lignin valorization harbors interesting opportunities, e. g., mild and eco-friendly process conditions (Cagide and Castro-Sowinski, 2020; Picart et al., 2015), additional efforts are still needed to bring this to a next level of industrial feasibility. For instance, *in vitro* application of lignin depolymerizing enzymes is limited by a commonly observed dimerization and repolymerization, a problem which might eventually be overcome in the future by reductase enzymes (Bugg et al., 2020), or the supplementation of nucleophilic compounds (Cagide and Castro-Sowinski, 2020).

Table 1

Lignin-valorization using metabolically engineered cell factories. The bacteria's substrate spectrum of selected natural and technical lignin-relevant aromatics is depicted as follows: "-" – verified no growth, "?" – unknown to authors, brown hexagon – metabolism as sole carbon/energy source likely, based on *in vivo* metabolite analysis, *in vitro* conversion, *in silico* pathway prediction, yellow hexagon – natural conversion demonstrated *in vivo* (biotransformation, or sole carbon/energy source), orange hexagon – metabolically engineered pathway for substrate conversion, [a]: substrate of parent strain with TOL-plasmid. Enzyme abbreviations: catalase (Cat), DyP - type peroxidase (DyP), glutathione-S-transferase (GST), laccase (Lac), multi-copper oxidase (McO), peroxidase (PeO), putrescine oxidase (PuO), sarcosine oxidase (Sox). Product spectrum is depicted as circles: acids (medium pink) – lactate (LAC), pyruvate (PYR); aromatics (light grey) – vanillin (VIN), styrene-derivatives (STY); dicarboxylic acids (pink) – adipate (ADA), itaconate (ITA), β-ketoadipate (β-KA), *cis, cis*-muconate (MA), methylated *cis, cis*-muconate variants (MA^M), 2-pyrone-4,6-dicarboxylate (PDC); pyridines (light pink) – pyridine-2,4-dicarboxylate (PDA^4), pyridine-2,5-dicarboxylate (PDA^5); storage compounds (dark pink) – polyhydroxyalkanoates (PHA); others (dark grey) – amino acids (AMA), carotenoids (CAR). The classification of potential applications is based on (Weimer et al., 2020). The following references were used to collect the information: *Amycolatopsis* sp. strain ATCC 39116 (formerly *Streptomyces setonii; Streptomyces griseus* 75iv2) (Barton et al., 2018; Brown et al., 2012, 2011; Fleige et al., 2016, 2013; Max et al., 2012; Meyer et al., 2018; Pometto III et al., 1981; Salvachúa et al., 2015; Sutherland et al., 1983), *Corynebacterium glutamicum* (formerly *Micrococcus glutamicus*) (Becker et al., 2018; Kallscheuer et al., 2016; Kinoshita et al., 1957; Lee et al., 2010; Merkens et al., 2005; Shen et al., 2005; Udaka, 1960), *Novophingobium aromaticivorans* (formerly *Sphingomonas* sp. strain F199) (Cecil et al., 2018; Fredrickson et al., 1991, 1995; Kontur et al., 2018; Perez et al., 2019; Romine et al., 1999), *Pseudomonas putida* KT2440 (Ahmad et al., 2010; Bagdasarian et al., 1981; Elmore et al., 2021; Graf and Altenbuchner, 2014; Granja-Travez and Bugg, 2018; Jiménez et al., 2002; Johnson and Beckham, 2015; Kohlstedt et al., 2018; Linger et al., 2014; Machovina et al., 2019; Murray et al., 1972; Nakazawa, 2002; Niu et al., 2020; Nogales et al., 2011; Notonier et al., 2021; Okamura-Abe et al., 2016; Salvachúa et al., 2015; Tumen-Velasquez et al., 2018; Vardon et al., 2015; Wada et al., 2021; Williamson et al., 2020; Worsey and Williams, 1975), *Rhodococcus jostii* RHA1 (formerly *Rhodococcus* sp. strain RHA1) (Ahmad et al., 2011, 2010; Chen et al., 2012; Chen and Wan, 2021; Fetherolf et al., 2020; Li et al., 2019b; Mycroft et al., 2015; Otani et al., 2014; Patrauchan et al., 2008, 2005; Sainsbury et al., 2013; Salvachúa et al., 2015; Seto et al., 1995; Spence et al., 2020; Szőköl et al., 2014; Takeda et al., 2010; Vignali et al., 2018), *Sphingobium* sp. strain SYK-6 (formerly *Sphingomonas* (*Pseudomonas*) *paucimobilis* SYK-6) (Higuchi et al., 2020; Masai et al., 2002, 2004, 2007a, 2007b; Meux et al., 2012; Nishikawa et al., 1998; Sonoki et al., 2018).

Bacteria						
	Gram-negative *Proteobacteria*			Gram-positive *Actinobacteria*		
	***P. putida* KT2440**	***Sphingobium* sp. SYK-6**	***N. aromatici-vorans***	***Amycola-topsis* sp.**	***C. glutamicum***	***R. jostii* RHA1**
						
Isolation site	Derivative of *P. arvilla* mt-2 (soil)	Pulp-bleaching wastewater	Coastal plain subsurface sediments	Soil	Soil	Lindane-contaminated soil
Lignin degrading/ accessory enzymes	McO; Lac; DyP	GST	GST	Cat; DyP; Lac		DyP; Lac; PeO; Sox; PuO; Cat
Substrate spectrum						
Benzoate	⬢	?	⬢	⬢	⬢	⬢
Caffeate	⬢	⬢	?	⬢	⬢	⬢
Catechol	⬢	?	?	⬢	⬢	⬢
p-Coumarate	⬢	⬢	⬢	⬢	⬢	⬢
m-Cresol	⬢	?	-	?	?	?
o-Cresol	⬢	?	-	⬢	?	?
p-Cresol	⬢	?	⬢	?	⬢	?
Cinnamate	-	?	?	⬢	⬢	?
Ferulate	⬢	⬢	⬢	⬢	⬢	⬢
Gallate	⬢	⬢	⬢	?	?	?
Guaiacol	⬢	?	?	⬢	?	⬢
p-Hydroxybenzoate	⬢	?	⬢	⬢	⬢	⬢
Phenol	⬢	?	?	⬢	⬢	⬢
Protocatechuate	⬢	⬢	⬢	⬢	⬢	⬢
Sinapate	?	⬢	⬢	?	?	-
Syringate	⬢	⬢	⬢	?	?	?
Syringol	⬢	?	?	?	?	?
m-Toluate	-[a]	?	⬢	?	?	?
o-Toluate	-	?	?	?	?	?
p-Toluate	-[a]	?	⬢	?	?	?
Toluene	-[a]	?	⬢	⬢	?	⬢
Vanillate	⬢	⬢	⬢	⬢	⬢	⬢
Vanillin	⬢	⬢	⬢	⬢	⬢	⬢
m-Xylene	-[a]	?	⬢	?	?	-
o-Xylene	-	?	⬢	?	?	⬢
p-Xylene	-[a]	?	⬢	?	?	-
Product spectrum						
Food and Feed	VIN STY ADA PYR LAC PHA			VIN STY	AMA	VIN CAR
Health and Hygiene	VIN ADA LAC PHA			VIN	AMA	VIN CAR
Packaging and Housing	VIN STY LAC ITA MA MA[M] ADA βKA PDC PHA	MA	PDC	VIN STY MA MA[M]	MA	VIN PDA[4] PDA[5]

2.3.2. Chemical depolymerization of lignin

Variable chemical depolymerization methods are available for lignin, which often rely on high pressure, high temperature, different catalysts and can be differentiated based on the underlying major cleavage mechanism (Abdelaziz et al., 2016; Schutyser et al., 2018; Van den Bosch et al., 2018). Regarding the various factors that influence monomer yield in addition to the depolymerization method, e.g., lignin isolation strategy, lignin source, a straightforward comparison of the different methods was pointed out as difficult (Schutyser et al., 2018). Nevertheless, the aromatic product spectrum is apparently closely

related to the gravity of the depolymerization (e.g., mild/harsh treatment), as well as the structure of the lignin (e.g., condensed/native like) (Schutyser et al., 2018), whereby mild depolymerization methods targeting at β-ethers are suggested to be more suitable for lignins where a high degree of the native β-*O*-4 linkages is maintained (Rinaldi et al., 2016).

In an exemplary study, the aromatic monomers in lignin liquors, obtained by mild base-catalyzed depolymerization from corn-stover lignin under different process conditions (temperature, NaOH concentration), were quantified (Rodriguez et al., 2017). Next to *p*-coumarate, which was found to be the dominant monomer, other aromatics were detected in the lignin liquor: the second most abundant ferulate, different benzoates (e.g., syringate, vanillate, *p*-hydroxybenzoate), and aldehydes (e.g., vanillin, *p*-hydroxybenzaldehyde) at lower concentrations (Rodriguez et al., 2017). This reported product spectrum corresponds well to the "natural" aromatic spectrum, early identified in soils (Shindo et al., 1978; Whitehead, 1964), and the suitability of the obtained lignin liquors for actual biological conversion was tested for different microbes (Rodriguez et al., 2017).

To overcome the highly compacted structure and rigid C–C bonds of technical lignins, mild cleavage methods are often ineffective, and rather harsh conditions (e.g., high temperature) are required (Rinaldi et al., 2016), which have the disadvantage of supporting repolymerization reactions, competitory to monomer yield (Schutyser et al., 2018). In pyrolysis, lignin is thermally degraded (450–600 °C) in the absence of oxygen (Schutyser et al., 2018). Common aromatic monomers obtained from pyrolysis are *inter alia* (alkylated) catechols and (alkylated) phenols (De Wild et al., 2014; Kawamoto, 2017; Schutyser et al., 2018), which according to the current understanding can be traced back to the effect of pyrolysis temperature on lignins (Kawamoto, 2017). Whereas in primary pyrolysis (200–400 °C) aromatic methoxy groups are quite stable, in secondary pyrolysis, temperatures exceeding 400 °C give rise to aromatics with short, saturated alkyl side-chains or no substitution, caused by a thermal cracking of side-chain C–C bonds (Kawamoto, 2017). Considering that depolymerization of compact lignins rather yields methoxyphenols with short side-chains (Schutyser et al., 2018), it appears noteworthy, that for instance bacterial degradation of Kraft lignin with *Paenibacillus* sp., *Aneurinibacillus aneurinilyticus* and *Bacillus* sp. resulted in strain-specific aromatics, also with larger side-chains, including for instance ferulate and cinnamate (Raj et al., 2007).

3. The who's who in microbial lignin metabolism

Microbial lignin degradation comprises extracellular depolymerization of the polymer and intracellular metabolism of the lignin-derived aromatics (Tian et al., 2014). Over the years, a collaboration of quite diverse microbes has been found to contribute to lignin degradation (Brink et al., 2019). Regarding the technical hurdles aggravating lignin valorization, it is of course tempting to transfer the naturally proven scheme of microbial lignin valorization on industrial level, but at this stage not all of the involved microbes appear equally suitable for biotechnological application (Beckham et al., 2016; Bugg et al., 2021).

3.1. Bacteria

Most works (including the success stories given below) rely on bacteria (Table S1, Supplementary File 2), pointing out their metabolic capabilities as the current heartbeat to unleash the potential of residual lignins. Bacteria that are naturally associated with lignin depolymerization or the degradation of aromatic compounds belong to six phyla (*Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, Spirochaetes*) (Brink et al., 2019). Hereby, natural solutions for anaerobic aromatic metabolism (Carmona et al., 2009; Fuchs et al., 2011; Harwood et al., 1998), have so-far remained of minor interest for lignin valorization, rather focusing on the classical aerobic aromatic degradation pathways (Fuchs et al., 2011).

Prominently, bacteria that naturally degrade lignin and/or metabolize aromatics have emerged as most potent hosts for the lignin industry (Table 1). Especially, the domineering phylum of *Proteobacteria* (Brink et al., 2019) has come forward with the important host *Pseudomonas putida* KT2440 and the model bacterium *Sphingobium* sp. strain SYK-6 (Table 1). Together with the *Actinobacteria Rhodococcus jostii* RHA1 and *Amycolatopsis* sp. strain ATCC 39116 they make up a forceful spearhead for lignin valorization strategies (Table 1). Considering also, the more recent and promising applications of *Novosphingobium aromaticivorans* DSM 12444 (Perez et al., 2019) and *Corynebacterium glutamicum* (Becker et al., 2018), it seems that the variety of fine-tuned, well-orchestrated aromatic pathways promotes an in-built superiority to withstand the demanding nature of the lignin polymer and its monomers. This trait apparently would be demanding and difficult, if not impossible, to program *de novo* to the same performance in a completely unskilled microbe. It is worth mentioning that also the archetypical prokaryotic expression system *Escherichia coli* (Yin et al., 2007), was successfully used for the conversion of aromatics into value-added products (Chen et al., 2018; L. Li et al., 2019a). However, the use of engineered *E. coli* seems hardly workable on the long run with regards to the aspired utilization of complex substrate mixtures in lignin hydrolysates (Abdelaziz et al., 2016; Beckham et al., 2016), consequently requiring the incorporation of multiple heterologous pathways.

As mentioned earlier (see **2.3.1**), the field of research coping with lignin depolymerization in general, and also, the exact contribution of bacteria to tackle this biopolymer, is still facing several unknowns (Bugg et al., 2020). There is, however, an increasing evidence that bacteria might play a far more dominant role in lignin depolymerization than previously imagined (Ahmad et al., 2010; Brown et al., 2011; Brown and Chang, 2014; Bugg et al., 2011b; Janusz et al., 2017; Tian et al., 2014). Notably, bacteria were actually quite early reported to be capable of lignin degradation (Crawford et al., 1983; Deschamps et al., 1981), with the first bacterial lignin peroxidase reported in 1988 (Ramachandra et al., 1988). Nowadays, the recurringly demonstrated power of bacterial enzymes for technical lignin degradation, e.g., from *Cupriavidus basilensis* B-8 (Shi et al., 2013), *Pandoraea* sp. B-6 (Liu et al., 2019), *P. putida* NX-1 (Xu et al., 2018), and *Streptomyces* sp. S6 (Riyadi et al., 2020) suggests more to come in the future. Noteworthy, in an extensive screening, fourteen bacteria were investigated for their capabilities to depolymerize lignin *via* different approaches (Salvachúa et al., 2015). Among the tested strains, *Amycolatopsis* sp. strain ATCC 39116, the two *P. putida* strains KT2440 and mt-2, as well as *R. jostii* RHA-1, stood out among the best performers (Salvachúa et al., 2015), adding to their established role as key players (Table 1). Also, the discovery of outer membrane vesicles, which are suggested to play a role in an extracellular catabolism of aromatic compounds (Choi et al., 2014; Salvachúa et al., 2020b), adds significantly to the fascination for harnessing bacteria in a lignin valorization context.

3.2. Fungi

Wood-rotting fungi have been long investigated regarding their capabilities to degrade lignocellulosic biomass (Eslyn et al., 1975; Henderson, 1955; Higuchi et al., 1955). Generally, wood-rotting fungi are classified as (i) white-rot, (ii) brown-rot, and (iii) soft-rot, considering that their different lignocellulose degradation mechanisms, mirror in the wood's appearance after progressed decay (Andlar et al., 2018; Janusz et al., 2017). White-rot fungi, largely belonging to the *Basidiomycota*, are most interesting based on their remarkable capability to achieve lignin's total degradation into carbon dioxide and water (Janusz et al., 2017). The early mechanisms of lignin degradation were clarified with *Phanerochaete chrysosporium,* leading to the discovery of lignin peroxidase and manganese peroxidase (Bugg et al., 2011a), and this fungus is characterized by a highly pronounced lignin degradation capability (Ahmad et al., 2010). Recently, it was shown that two model white-rot fungi, *Trametes versicolor* and *Gelatoporia subvermispora,* catabolize

lignin degradation products and obviously possess intracellular aromatics pathways (del Cerro et al., 2021). Admittedly, harnessing fungi or fungal enzymes for lignin valorization is impaired by slow growth rates, demanding growth conditions (del Cerro et al., 2021), and a lack in tools for genetic manipulation and protein expression (Bugg et al., 2011b). However, the last years of research gave reason to hope for improvements in genetic engineering of white-rot fungi, opening up their impressive capabilities for lignin valorization in the future (Bugg et al., 2021).

3.3. Yeasts

While common biotechnological yeasts such as *Hansenula, Pichia,* and *Saccharomyces* (Yin et al., 2007) play a minor role in aromatics degradation (Mills et al., 1971), other yeasts were reported to exhibit a broad aromatic substrate spectrum (Bergauer et al., 2005; Gupta et al., 1986; Mills et al., 1971; Sampaio, 1995), and partially, even lignin degradation capabilities (Elena and Božena, 2001; Hainal et al., 2012; Sláviková et al., 2002). Especially, the oleaginous *Rhodotorula* clade was recurringly mentioned to provide promising aromatic degraders (Bergauer et al., 2005; Gupta et al., 1986; Hainal et al., 2012; Huang et al., 1993; Mills et al., 1971; Sampaio, 1995; Yaegashi et al., 2017; Yaguchi et al., 2020). Surprisingly, the evident potential has been largely left unnoticed and only in the last years, the interest in yeasts for lignin valorization seems to revive (Yaegashi et al., 2017; Yaguchi et al., 2017, 2020).

3.4. Archaea

Selected archaea are capable of aromatics assimilation (Emerson et al., 1994). Recently, the capability to degrade aromatic compounds under anaerobic conditions was suggested for a novel archaeal phylum, designated as *Hermodarchaeota* (Zhang et al., 2021). Also, laccase-like enzymes, relevant for lignin degradation, have been identified in different archaea (Tian et al., 2014) and reports suggest, that archaea might play a so-far undiscovered role in degrading the lignin polymer in marine sediments (Yu et al., 2018). Thus, it can be assumed, that more archaeal representatives will be discovered, leading to a better understanding of their role in lignin degradation and assimilation, which might also be of value for the cloning of promising enzymes into other hosts.

4. Metabolic strategies for lignin degradation

4.1. Overarching routes for natural and technical lignin-derived aromatics

In the following, the metabolism of approximately 50 different "natural" and "technical" lignin-associated aromatics is covered (Fig. S1, Supplementary File 1), focusing on representative monomers obtained from biobased (see **2.3.1**) and chemical (see **2.3.2**) depolymerization. Generally, the metabolic strategies applied by different microorganisms to master the unruly character of aromatics, formed from depolymerized lignin, share the same pattern, independent from the oxygen preference (Díaz, 2004; Fuchs et al., 2011; Harwood and Parales, 1996).

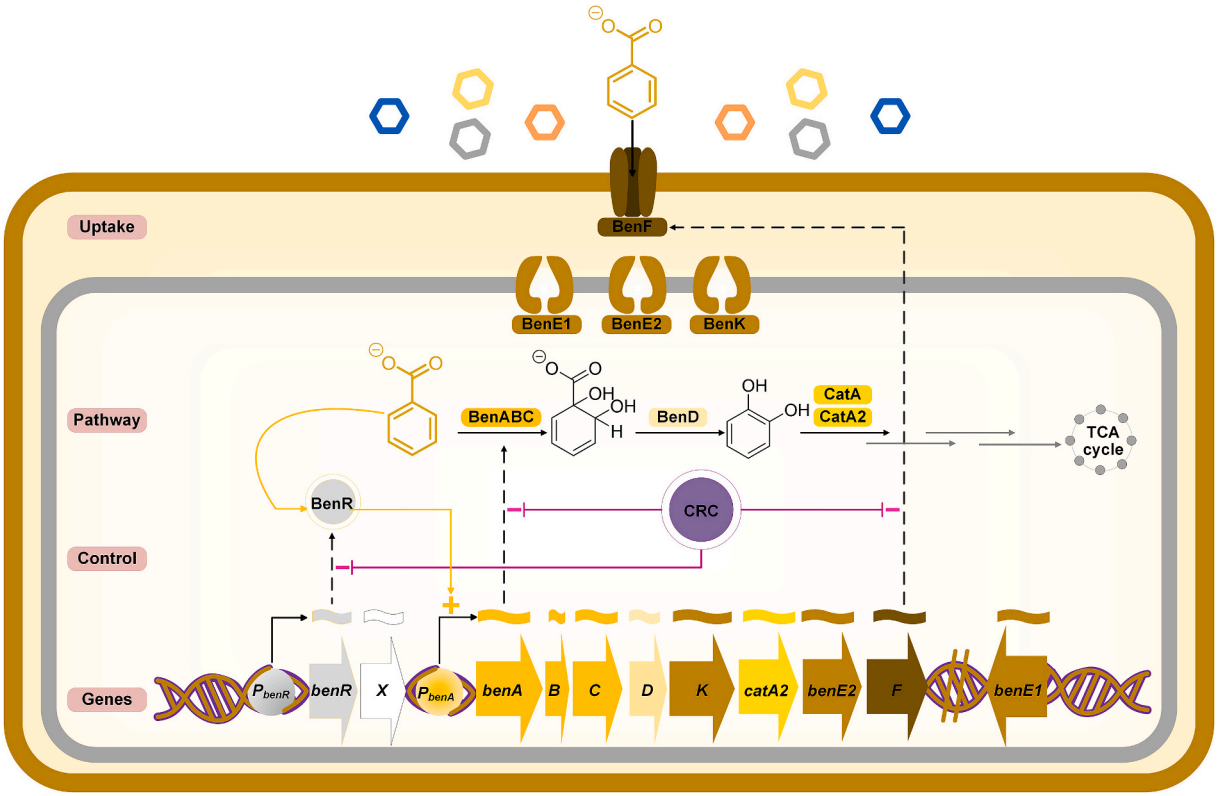


Fig. 2. Genetic architecture and control of benzoate degradation in *P. putida* KT2440. The *ben*-cluster encodes for catabolic enzymes (BenABC: benzoate-dioxygenase, orange; BenD: benzoate diol dehydrogenase, light orange; a second catechol-1,2-dioxygenase CatA2, yellow; in addition to the catechol-1,2-dioxygenase, associated with the *cat*-cluster (not shown here)); a regulator BenR, grey; the transporters BenE1, BenE2, BenK, light ochre; the porin BenF, brown; and BenX: a protein with unknown function, white (Jiménez et al., 2002, 2014; Nishikawa et al., 2008; van Duuren et al., 2011). Locally, the transcriptional regulator BenR (Jiménez et al., 2002) activates (+) the expression of the *ben*-cluster in the presence of benzoate, as shown for the respective orthologue in *P. putida* PRS2000 (Cowles et al., 2000). On global level, the translational repressor Crc (violet) inhibits (-) the translation (dashed arrows) of *benR*, *benF* and *benA* mRNAs (Hernández-Arranz et al., 2013). The *benE1* gene, which is involved in benzoate uptake (Nishikawa et al., 2008), is not part of the BenR-regulon (Hernández-Arranz et al., 2013). Symbols: promoters – circles, genes – arrows, mRNAs – waves, regulators – concentric circles, transporters – two crescents, porin – tapered cylinder. Parts of the figure were adapted from previous works (Borrero-de Acuña et al., 2020; Hernández-Arranz et al., 2013; Kamimura et al., 2017b).

Structurally variable aromatics are channeled by upper/peripheral pathways into selected central intermediates (Díaz, 2004; Fuchs et al., 2011) (Fig. 3). After overcoming the aromaticity of these compounds by ring cleavage, further lower/central pathways channel the intermediary metabolites towards central metabolism (Díaz, 2004; Fuchs et al., 2011), thus, making them available for cell growth, maintenance, and secondary metabolism (Li and Zheng, 2020) (Fig. 4). Additionally, decarboxylases interconnect the various catabolic pathways and upgrade the converging (linear) routes into a complex, versatile network (Crawford and Olson, 1978). This architecture, spanning across various aromatics, is the foundation for current and future success stories relying on a bacterial valorization of lignin and aromatic compounds (Table S1, Supplementary File 2), and is pictorially labelled as "biological funneling" (Beckham et al., 2016; Linger et al., 2014).

4.2. Upper pathways and sequential oxidations

The biotransformation strategy in the upper pathways is directed by

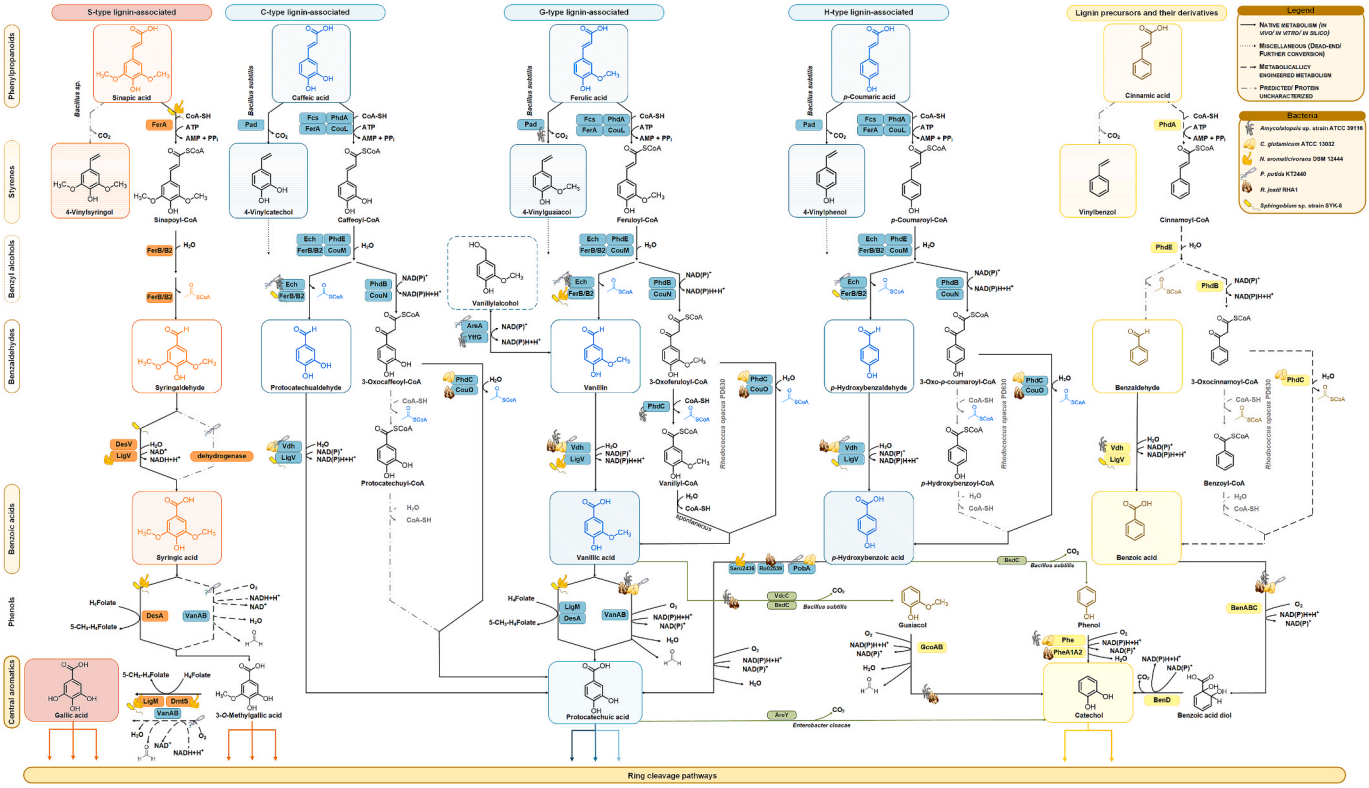


Fig. 3. Upper pathways for the funneling of structural diverse aromatics into few central aromatics. Lignin depolymerization products may enter the upper pathways at different levels. Mild alkaline treatment was shown to predominantly yield phenylpropanoids (e.g., *p*-coumarate, ferulate) (Karp et al., 2016; Rodriguez et al., 2017), whereas microbial depolymerization gives rise to a couple of differently substituted aromatic aldehydes, acids or guaiacol (Bugg et al., 2011a; Higuchi et al., 2020; Lancefield et al., 2016). Benzaldehyde: Vanillin dehydrogenase (Vdh (Fleige et al., 2013), LigV (Masai et al., 2007b)), Benzoate: Benzoate dioxygenase (BenABC), Benzoate diol dehydrogenase (BenD) (Jiménez et al., 2002; Park and Kim, 2003; Patrauchan et al., 2005; Shen et al., 2012), Guaiacol: Guaiacol-*O*-demethylase (GcoAB) (Fetherolf et al., 2020; Mallinson et al., 2018), *p*-Hydroxybenzaldehyde: Vanillin dehydrogenase (Vdh (Ding et al., 2015; Jiménez et al., 2002; Sainsbury et al., 2013), LigV (Masai et al., 2007b)), *p*-Hydroxybenzoate: *p*-Hydroxybenzoate-3-hydroxylase (PobA (Huang et al., 2008; Jiménez et al., 2002), Ro02539 (Montersino and van Berkel, 2012), Saro_2436 (Perez et al., 2019)), 3-*O*-Methylgallate: Vanillate/3-*O*-Methylgallate-*O*-demethylase (LigM) (Abe et al., 2005; Perez et al., 2021), *O*-demethylase (DmtS) (Perez et al., 2021), Vanillate *O*-demethylase (VanAB) (Notonier et al., 2021), Phenol: Phenol-Hydroxylase (PheA1A2 (Szőköl et al., 2014), Phe (Xiao et al., 2015), (Antai and Crawford, 1983)), Phenylpropanoids: *Amycolatopsis* sp. strain ATCC 39116 - Feruloyl-CoA synthetase (Fcs), Enoyl-CoA hydratase/aldolase (Ech), Enoyl-CoA-hydratase (Ech2), 4-Hydroxy-3-methoxyphenyl-β-hydroxypropionyl-CoA-dehydrogenase (PhdB), 4-Hydroxy-3-methoxyphenyl-β-ketopropionyl-CoA dehydratase (PhdC) (Meyer et al., 2018), Ferulic acid decarboxylase (Max et al., 2012), *Bacillus subtilis* - Phenolic acid decarboxylase (Pad) (Cavin et al., 1998), *Bacillus* sp. - Sinapate decarboxylase (Ohta et al., 2012), *Corynebacterium glutamicum* - Acyl-CoA ligase (PhdA), Enoyl-CoA hydratase (PhdE), 3-Hydroxyacyl-CoA dehydrogenase (PhdB), 3-Oxoacyl-CoA ketohydrolase (PhdC) (Kallscheuer et al., 2016), *Novosphingobium aromaticivorans* - Feruloyl-CoA-synthetase (FerA), Feruloyl-CoA hydratase/lyase (FerB) (Cecil et al., 2018), *Rhodococcus jostii* RHA1 - *p*-Hydroxycinnamoyl-CoA-synthetase (CouL), *p*-Hydroxycinnamoyl-CoA hydratase (CouM), *p*-Hydroxyphenoxy-β-hydroxyacyl-CoA dehydrogenase (CouN), *p*-Hydroxyphenoxy-β-ketoacyl-CoA hydrolase (CouO) (Otani et al., 2014), *Sphingobium* sp. strain SYK-6 - Feruloyl-CoA-synthetase (FerA), Feruloyl-CoA hydratase/lyase (FerB; FerB2) (Masai et al., 2002), *Pseudomonas putida* KT2440 - Feruloyl-CoA synthetase (Fcs), *p*-Hydroxycinnamoyl-CoA hydratase/aldolase (Ech) (Jiménez et al., 2002; Plaggenborg et al., 2003), *Rhodococcus opacus* PD630 (Cai et al., 2020). Regarding the widespread promiscuity in phenylpropanoid degradation, we took the liberty to add three pathways, (i) caffeate degradation *via* protocatechuyl-CoA, (ii) cinnamate degradation *via* benzaldehyde, the latter matching the metabolites observed by (Sutherland et al., 1983), (iii) decarboxylation of cinnamate into vinylbenzol. Protocatechualdehyde: Vanillin dehydrogenase (Vdh (Ding et al., 2015; Jiménez et al., 2002), LigV (Masai et al., 2007b)), Syringaldehyde: Syringaldehyde dehydrogenase (DesV) (Kamimura et al., 2017a), Vanillin dehydrogenase (LigV) (Cecil et al., 2018; Masai et al., 2007b), uncharacterized (Notonier et al., 2021), Syringate: tetrahydrofolate-dependent Syringate *O*-demethylase (DesA) (Masai et al., 2004; Perez et al., 2021), Vanillate-*O*-demethylase (VanAB) (Notonier et al., 2021), Vanillate: Vanillate-*O*-demethylase (VanAB) (Chen et al., 2012; Jiménez et al., 2002; Merkens et al., 2005; Meyer et al., 2018), Vanillate/3-*O*-Methylgallate-*O*-demethylase (LigM) (Abe et al., 2005; Perez et al., 2021), Vanillate/Syringate-*O*-demethylase (DesA) (Abe et al., 2005; Nishikawa et al., 1998; Perez et al., 2021), Vanillin: Vanillin dehydrogenase (Vdh (Chen et al., 2012; Ding et al., 2015; Fleige et al., 2013; Plaggenborg et al., 2003), LigV (Cecil et al., 2018; Masai et al., 2007b)), Vanillin reductase (AreA (García-Hidalgo et al., 2020), YtfG (Meyer et al., 2018)), Coupling reactions: Protocatechuate decarboxylase (AroY) (Vardon et al., 2015), Vanillate/*p*-Hydroxybenzoate decarboxylase (BsdC) (Graf et al., 2016; Lupa et al., 2008), Vanillate decarboxylase (VdcC) (Meyer et al., 2017; Spence et al., 2020).

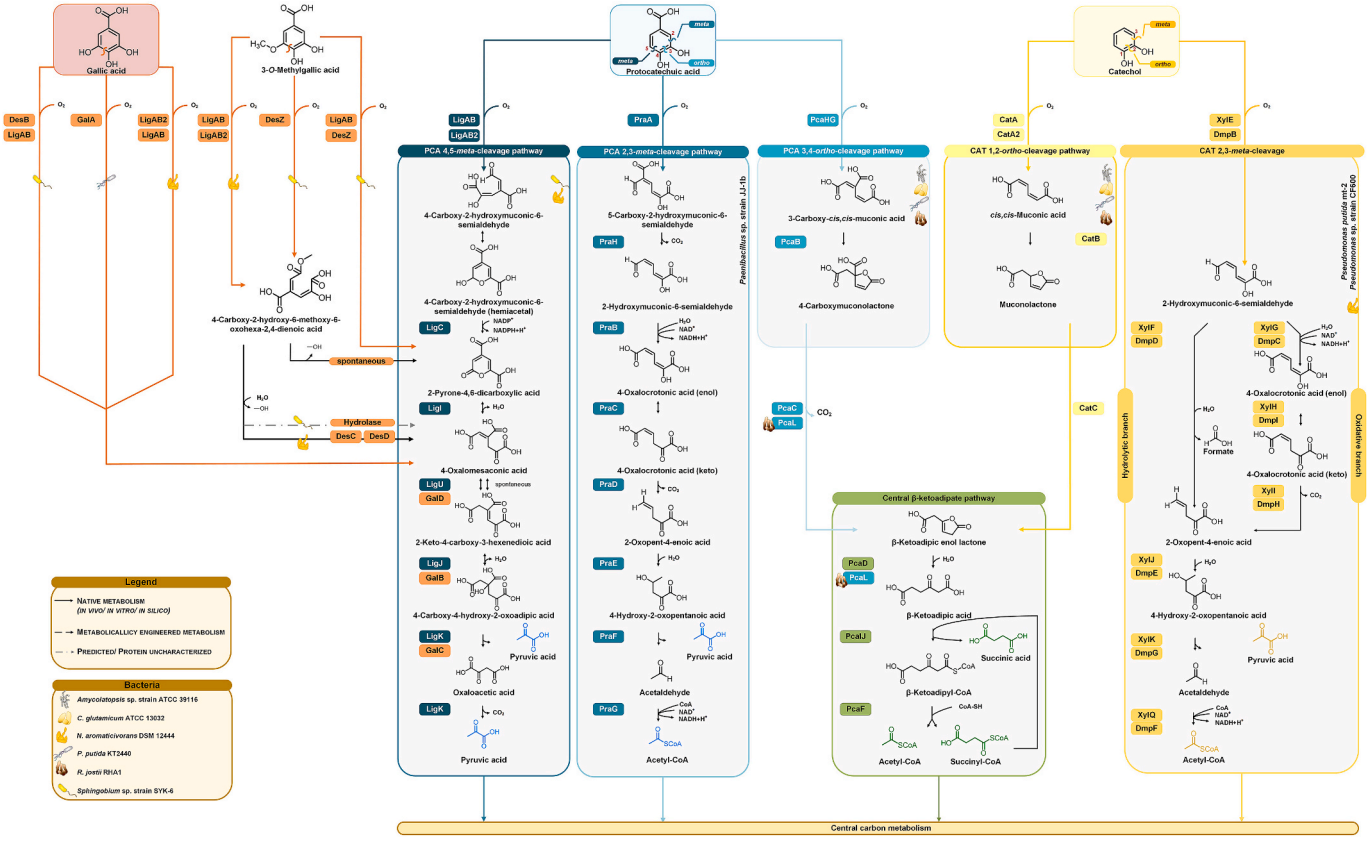


Fig. 4. Ring-cleavage and lower pathways for the degradation of central aromatic intermediates. Gallate cleavage: *Novosphingobium aromaticivorans* - ring-cleavage dioxygenases (LigAB, LigAB2) (Perez et al., 2021), *Pseudomonas putida* KT2440 - Gallate dioxygenase (GalA), 4-Oxalomesaconate keto-enol tautomerase (GalD), 4-Oxalomesaconate enol hydratase (GalB), 4-Carboxy-4-hydroxy-2-oxoadipate aldolase (GalC) (Nogales et al., 2011), Protocatechuate-3,4-dioxygenase (PcaHG) (not shown) (Notonier et al., 2021), *Sphingobium* sp. strain SYK-6 - Gallate dioxygenase (DesB), Protocatechuate-4,5-dioxygenase (LigAB) (Kasai et al., 2005), 3-*O*-Methylgallate cleavage: *Novosphingobium aromaticivorans* – ring-cleavage dioxygenases (LigAB, LigAB2) (Perez et al., 2021), 4-Carboxy-2-hydroxy-6-methoxy-6-oxohexa-2,4-dienoate methyl-esterase (DesCD) (Cecil et al., 2018), spontaneous disintegration of 4-Carboxy-2-hydroxy-6-methoxy-6-oxohexa-2,4-dienoate (Perez et al., 2019), *Sphingobium* sp. strain SYK-6 - 3-*O*-Methylgallate 3,4-dioxygenase (DesZ), Protocatechuate-4,5-dioxygenase (LigAB) (Kasai et al., 2004), 4-Carboxy-2-hydroxy-6-methoxy-6-oxohexa-2,4-dienoate hydrolase (unknown) (Araki et al., 2020), spontaneous disintegration of 4-Carboxy-2-hydroxy-6-methoxy-6-oxohexa-2,4-dienoate (Kasai et al., 2004), Protocatechuate-4,5-*meta*-cleavage: *Novosphingobium aromaticivorans* - (Cecil et al., 2018), ring-cleavage dioxygenase (LigAB2) (Perez et al., 2021), *Sphingobium* sp. strain SYK-6 - Protocatechuate-4,5-dioxygenase (LigAB) (Noda et al., 1990), 4-Carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase (LigC) (Masai et al., 2000), 2-Pyrone-4,6-dicarboxylate hydrolase (LigI) (Masai et al., 1999), 4-Oxalomesaconate delta isomerase (LigU) (Hogancamp and Raushel, 2018), 4-Oxalomesaconate hydratase (LigJ) (Hara et al., 2000), 4-Carboxy-4-hydroxy-2-oxoadipate aldolase/Oxaloacetate decarboxylase (LigK) (Hara et al., 2003), Protocatechuate-2,3-*meta*-cleavage: Protocatechuate 2,3-dioxygenase (PraA), 5-Carboxy-2-hydroxymuconate-6-semialdehyde decarboxylase (PraH), 2-Hydroxymuconate-6-semialdehyde dehydrogenase (PraB), 4-Oxalocrotonate tautomerase (PraC), 4-Oxalocrotonate decarboxylase (PraD), 2-Oxopent-4-enoate hydratase (PraE), 4-Hydroxy-2-oxovalerate aldolase (PraF), Acetaldehyde dehydrogenase (PraG) (Kasai et al., 2009), Protocatechuate-3,4-*ortho*-cleavage (Protocatechuate branch β-ketoadipate pathway): Protocatechuate-3,4-dioxygenase (PcaHG), β-Carboxy-*cis,cis*-muconate cycloisomerase (PcaB), γ-Carboxy-muconolactone decarboxylase (PcaC) (Harwood and Parales, 1996; Jiménez et al., 2002; Meyer et al., 2018; Patrauchan et al., 2005; Shen et al., 2012), Central branch β-ketoadipate pathway: β-Ketoadipate enol-lactone hydrolase (PcaD), β-Ketoadipate succinyl-CoA transferase (PcaIJ), β-Ketoadipyl-CoA thiolase (PcaF) (Harwood and Parales, 1996; Jiménez et al., 2002; Meyer et al., 2018; Patrauchan et al., 2005; Shen et al., 2012), bifunctional γ-Carboxymuconolactone decarboxylase/enol-lactone hydrolase (PcaL) (Patrauchan et al., 2005), Catechol-1,2-*ortho*-cleavage (Catechol branch β-ketoadipate pathway): Catechol-1, 2-dioxygenase (CatA), *cis, cis*-Muconate cycloisomerase (CatB), Muconolactone isomerase (CatC) (An et al., 2001; Harwood and Parales, 1996; Jiménez et al., 2002; Park and Kim, 2003; Patrauchan et al., 2005; Shen et al., 2012), Catechol-1,2-dioxygenase (CatA2) (Jiménez et al., 2002; van Duuren et al., 2011), Catechol-2, 3-*meta*-cleavage: Catechol-2,3-dioxygenase (XylE, DmpB), 2-Hydroxymuconic semialdehyde hydrolase (XylF, DmpD), 2-Hydroxymuconic semialdehyde dehydrogenase (XylG, DmpC), 4-Oxalocrotonoate tautomerase (XylH, DmpI), 4-Oxalocrotonoate decarboxylase (XylI, DmpH), 2-Oxopent-4-enoate hydratase (XylJ, DmpE), 4-Hydroxy-2-oxalovalerate aldolase (XylK, DmpG), Aldehyde dehydrogenase (XylQ, DmpF) (Cecil et al., 2018; Franklin et al., 1981; Harayama and Rekik, 1990; Powlowski and Shingler, 1994; Romine et al., 1999).

oxidations (Fig. 3). Hereby, enzymes mediating hydroxylation (Kargegoudar and Kim, 2000; Shingler et al., 1989), and *O*-demethylation (Fetherolf et al., 2020; Kamimura et al., 2017b; Machovina et al., 2019; Mallinson et al., 2018) serve as the central arms in the upper pathways. Notably, to cope with the sheer mass of aromatics, bacteria economically make use of enzymes, which are featured by a broader substrate specificity than those of the lower, ring cleavage pathways, likewise allowing a flexible degradation of structurally similar aromatics in one microorganism (Díaz et al., 2013; van der Meer et al., 1992).

4.3. Lower pathways and aromatic ring cleavage

Staying with the picture of "biological funneling", the lower pathways form the "stem" of the funnel (Kohlstedt et al., 2018) (Fig. 4) with protocatechuate and catechol as major hubs (Abdelaziz et al., 2016; Harwood and Parales, 1996), followed by other central intermediates in more specialized pathways (Fig. S1, Supplementary File 1). As aromatics are featured by a limited chemical reactivity, overcoming the stabilizing resonance energy for ring cleavage is the biggest challenge for microbial degradation (Fuchs et al., 2011; Harwood and Parales, 1996). In nature,

ring cleavage is largely an oxygen-dependent process (Abdelaziz et al., 2016), and, in the lower pathways, is catalyzed by dioxygenases, which in contrast to the monooxygenases dominating the upper pathways, incorporate both and not only one of the oxygen atoms into their substrate (Fuchs et al., 2011). In general, depending on the host and as a trait of taxonomic significance (Beckham et al., 2016; Bugg et al., 2011a; Crawford, 1975; Dagley et al., 1968; Ni et al., 2013), protocatechuate and catechol are cleaved at different positions on the ring by intradiol (*ortho*) or extradiol (*meta*) dioxygenases (Harwood and Parales, 1996) (Fig. 4). In terms of completeness, based on gentisate substitution, gentisate-1,2-dioxygenase catalyzes the third possible type of ring cleavage, namely *para*-ring cleavage (Harwood and Parales, 1996) (Fig. S1, Supplementary File 1).

From a systems metabolic engineering viewpoint, it is helpful, that the evident metabolic diversity for the upper pathways continues to a certain extent with regards to the lower pathways, providing vast opportunities for different biotransformation strategies (see **section 7**). What's even more, making use of the naturally available ring cleavage routes for metabolic engineering is a valuable adjusting screw for the production performance because of a varying precursor supply (Borrero-de Acuña et al., 2020; Johnson and Beckham, 2015), as well as a different carbon dioxide and reducing equivalent formation (Johnson and Beckham, 2015).

4.4. Design principle of aromatic gene clusters

The understanding of the genetics behind aromatics catabolism is a pre-requisite to overcome undesired regulation mechanisms and install designed pathway modules towards streamlined aromatics cell factories. Genes involved in bioconversion of aromatic compounds are usually organized in clusters, displaying functionally distinct units subjected to precise control (Díaz et al., 2013; Jiménez et al., 2002). Although differing at the level of detail, the general layout is largely conserved. To illustrate the design principle and the achieved functionality, the *ben* cluster for benzoate catabolism of *P. putida* KT2440 (Jiménez et al., 2002), a well-studied chassis strain (Weimer et al., 2020) with distinct applications in lignin valorization (Table 1), can serve as a prominent example. Four catabolic genes, namely *benABCD,* encode for the two enzymes that convert benzoate into catechol (Fig. 2). Catechol is then subjected to oxidative ring-cleavage by catechol-1,2-dioxygenase, encoded in most microorganisms by the *catA* gene, that is part of a separate *cat*-cluster (Jiménez et al., 2002). The *ben*-cluster of *P. putida* KT2440, however, additionally includes a *catA2* gene that shows similarity to c*atA* (Jiménez et al., 2002). CatA2 (van Duuren et al., 2011) acts as a "safety valve", when the strain encounters high concentrations of toxic catechol (Jiménez et al., 2014). Regarding transport, charged aromatics do not easily diffuse across the cell membrane (Mori et al., 2018; Vermaas et al., 2019), so that transport proteins for these ionized molecules function as a competitive advantage at low level concentrations (Harwood and Parales, 1996; Nichols and Harwood, 1997; Shen et al., 2012). Thus, catabolic genes are often located in direct proximity to genes encoding for aromatic uptake (Díaz et al., 2013), and were respectively also identified (Jiménez et al., 2002) and confirmed in the *ben*-cluster (Nishikawa et al., 2008). In correspondence to another *P. putida* strain, the *benF*-gene is generally assumed to encode for a benzoate-specific porin (Jiménez et al., 2002). Functional analysis though indicated, that BenF might also display a benzoate efflux pump (Nishikawa et al., 2008).

4.5. Genetic regulation and control

Generally, the expression of gene clusters for aromatics catabolism underlies diverse mechanisms, whereby the regulatory mechanisms of the upper pathways are even supposed to surpass those of the central pathways (Díaz et al., 2013). Aromatic operons usually contain regulatory genes that encode for pathway-specific transcriptional regulators (Díaz et al., 2013; van der Meer et al., 1992) to be independently transcribed from the structural genes (Harwood and Parales, 1996). The local regulators act as sensors for the accessibility of the respective aromatic carbon source, assuring that the pathway genes are expressed only in the presence of the assimilated compound (Brinkrolf et al., 2006; Powlowski and Shingler, 1994). The *benR*-gene in *P. putida* KT2440 is an orthologue of a gene found in *P. putida* PRS2000 (Jiménez et al., 2002), shown to encode for a transcriptional activator BenR (AraC/XylS family), necessary for benzoate degradation (Cowles et al., 2000). Superimposed to local control, global catabolite repression control, mediated by the translational repressor CRC, plays a pivotal role in aromatics catabolism (Hernández-Arranz et al., 2013; Morales et al., 2004) (Fig. 2). In *P. putida*, CRC inhibits the translation of *benR*-mRNA, thus reducing the level of BenR below the threshold necessary for maximal activation of the P_{benA} promoter (Hernández-Arranz et al., 2013; Moreno and Rojo, 2008). Further studies additionally suggest, that CRC not only interferes at the level of *benR*, but also inhibits translation of *benA* and *benF* mRNAs (Hernández-Arranz et al., 2013). Thus, CRC-mediated control proceeds through multiple checkpoints, likely not only affecting local regulators, but also uptake mechanisms and catabolic enzymes (Hernández-Arranz et al., 2013).

5. Metabolic pathways for lignin degradation

5.1. Upper degradation routes for "natural" lignin aromatics

A useful distinction of the upper pathways for degradation of "natural" lignin aromatics follows the well-known categorization of lignin based on its monomers, into S-, G-, H-type aromatics (Brink et al., 2019) (Fig. 1). Settled high up in the "biological funnel" of bacterial aromatics metabolism are phenylpropanoids (e.g., ferulate, *p*-coumarate, and sinapate) (Fig. 3), featured by a phenyl group and an unsaturated carbon propene tail (Kallscheuer et al., 2016). Predominantly *p*-coumarate, and in lower amounts ferulate, are found in different agricultural residues (Sun et al., 2002; Xu et al., 2005) and grasses (Yan et al., 2010), where they were soon suspected to be involved in cross-linkages between the lignin and polysaccharide fractions (Iiyama et al., 1990). Cleavage of the respective (alkali-labile) ester and (acid-labile) ether bonds and quantification of phenolic acids/aldehydes was shown to be possible either by saponification *via* alkaline hydrolysis at low (room temperature) and high temperatures (170 °C), respectively (Iiyama et al., 1990), or sequential mild alkaline and acid hydrolysis (Xu et al., 2005). All in all, this goes well in line with the high concentrations of *p*-coumarate observed in alkaline pretreatment liquors (Karp et al., 2016) or lignin liquors from base-catalyzed depolymerization (Rodriguez et al., 2017) obtained from corn stover.

In each following section, we will first focus on phenylpropanoid degradation, which follows a general mechanism. In a first step, bacteria shorten the aliphatic side chain (Kallscheuer et al., 2016; Rosazza et al., 1995), which involves, among other mechanisms, biotechnologically relevant routes of (i) non-oxidative decarboxylation into styrene derivatives (Mishra et al., 2014), (ii) CoA-dependent non-β-oxidation (Overhage et al., 1999), and (iii) CoA-dependent β-oxidation strongly resembling fatty acid β-oxidation (Plaggenborg et al., 2006). Notably, phenylpropanoids are a prime case for the substrate promiscuity of enzymes (Cavin et al., 1998; Kallscheuer et al., 2016; Masai et al., 2002; Meyer et al., 2018; Mitra et al., 1999; Otani et al., 2014; Yang et al., 2013), which depending on the respective pathway gives rise to a recurring pattern of differently substituted aromatic aldehydes, acids, or styrene-derivatives (Fig. 3). Several of these, e.g., vanillate, were both detected in studies focusing on biobased (Lancefield et al., 2016), as well as mild chemical lignin depolymerization (Rodriguez et al., 2017), pointing out the importance to comprehend the respective pathways for the bacterial upgrading of lignin polymers.

5.1.1. G-type aromatics – ferulate, vanillin, and vanillate

Ferulate. Due to the relevance of ferulate as substrate for microbial vanillin production (**Section 7.2**), its degradation pathways probably belong to the best researched ones (Priefert et al., 2001; Rosazza et al., 1995). Microbes have evolved several possibilities to degrade ferulate (Rosazza et al., 1995), which not necessarily exclude each other in a single bacterium (Max et al., 2012; Meyer et al., 2018) and are apparently activated depending on the cultivation type and energetic state of the cell (Contreras-Jácquez et al., 2020). Non-oxidative decarboxylation of ferulate, among the oldest known biotransformations of phenylpropanoids (Rosazza et al., 1995), seems to gain in biotechnological interest during the past few years (Mishra et al., 2014; Williamson et al., 2020). It takes place *via* an enzymatically promoted formation of a temporary quinoid intermediate, which is decarboxylated into 4-vinylguaiacol (Huang et al., 1994) and then converted into vanillin (Karmakar et al., 2000; Mishra et al., 2014), 4-ethylguaiacol (van Beek and Priest, 2000) or remains as a dead-end metabolite (Graf et al., 2016; Mpofu et al., 2019). Several bacteria (Mishra et al., 2014), most prominently different *Bacillus* sp. (Cavin et al., 1998; Degrassi et al., 1995; Graf et al., 2016; Jung et al., 2013; Karmakar et al., 2000; Lee et al., 1998; L. Li et al., 2019a; Mpofu et al., 2019), possess decarboxylases that accept ferulate as a substrate. In contrast, CoA-dependent non-β-oxidation and β-oxidation are initiated by conversion of ferulate into feruloyl-CoA (Fig. 3). The non-β-oxidative pathway is equally common in the phyla of *Actinobacteria* (Achterholt et al., 2000; Yang et al., 2013), and *Proteobacteria,* including *P. putida* KT2440 (Plaggenborg et al., 2003), and *Sphingobium* sp. strain SYK-6 (Masai et al., 2002). The apparently less common β-oxidation pathway is reported in two different variants that proceed *via* different CoA intermediates and can be distinguished based on different cleavage mechanisms yielding acetyl-CoA (Kallscheuer et al., 2016; Meyer et al., 2018; Otani et al., 2014). In both cases, vanillate is generated during ferulate degradation (see below for further conversion).

Vanillin. The benzaldehyde-derivative vanillin is rather toxic, requiring fast conversion to prevent its accumulation in the cell (Priefert et al., 2001). Vanillin is usually oxidized into vanillate by vanillin dehydrogenase, designated Vdh (Chen et al., 2012; Ding et al., 2015; Fleige et al., 2013; Nishimura et al., 2018) and LigV (Masai et al., 2007b). With exceptions (Di Gioia et al., 2011), respective deletion mutants retained their ability to convert vanillin, hinting at other dehydrogenases for vanillin metabolism (Fleige et al., 2013; Graf and Altenbuchner, 2014; Masai et al., 2007b; Plaggenborg et al., 2003, 2006). Several works reported the reduction of vanillin into vanillyl alcohol, a common detoxification mechanism (Brink et al., 2019), found in *Amycolatopsis* sp. (Achterholt et al., 2000; Fleige et al., 2016; Muheim and Lerch, 1999), *B. subtilis* (Graf et al., 2016), and different *Pseudomonas* sp. (Graf and Altenbuchner, 2014; Ravi et al., 2018). Considering vanillyl alcohol as an undesired by-product in vanillin production, elimination of the recently identified responsible enzymes quinone oxidoreductase YtfG in *Amycolatopsis* sp. strain ATCC 39116 (Meyer et al., 2018) and aldehyde reductase AreA in *P. putida* (García-Hidalgo et al., 2020), could thus, be regarded as future pathbreakers for vanillin manufacturing (Fig. 3).

Vanillate. As the last step in this branch, vanillate is demethylated into protocatechuate, relying on two systems, the more common two-component vanillate-*O*-demethylase VanAB (Brunel and Davison, 1988; Chen et al., 2012; Jiménez et al., 2002; Merkens et al., 2005; Nishimura et al., 2006) and the tetrahydrofolate-dependent *O*-demethylase LigM, that is also involved in 3-*O*-methylgallate metabolism (Abe et al., 2005) (Fig. 3). As often found in aromatic degradation, both NADH and NADPH can be used as electron donors by VanAB depending on the microbial origin (Hibi et al., 2005). Importantly, conversion of vanillate into protocatechuate by VanAB results in toxic formaldehyde (Hibi et al., 2005), whereas LigM transfers the methyl moiety to the tetrahydrofolate-mediated one carbon metabolism (Abe et al., 2005; Masai et al., 2004).

5.1.2. H-type-lignin aromatics – p-coumarate, p-hydroxybenzoate

***p*-Coumarate.** Based on the common promiscuous character of enzymes in the upper pathways (van der Meer et al., 1992), basically the same enzymatic routes are available for *p*-coumarate as for ferulate, resulting either in *p*-hydroxybenzoate (*via* different CoA-intermediates) (Jung et al., 2016; Kallscheuer et al., 2016; Masai et al., 2002; Otani et al., 2014; Yang et al., 2013), or in 4-vinylphenol (*via* decarboxylation) (Jung et al., 2013; L. Li et al., 2019a; van Beek and Priest, 2000) (Fig. 3).

***p*-Hydroxybenzoate.** Microbial conversion of *p*-hydroxybenzoate proceeds *via* different routes, which, interestingly, can, also, co-exist in one bacterium (Jung et al., 2016). Commonly, *p*-hydroxybenzoate is hydroxylated into the central intermediate protocatechuate by *p*-hydroxybenzoate-3-hydroxylase (Entsch and Van Berkel, 1995), which, depending on the microorganism, differs in its preference for NADPH and NADH (Huang et al., 2008; Jadan et al., 2001; Kasai et al., 2009). It is relevant to note, that even though most bacteria tend to degrade *p*-hydroxybenzoate *via* protocatechuate, other hydroxybenzoates result in different intermediates (Karegoudar and Kim, 2000). Here, different hydroxylases convert *o*-hydroxybenzoate (salicylate) into gentisate or catechol, and *m*-hydroxybenzoate into protocatechuate, 2,3-dihydroxybenzoate and gentisate, respectively (Karegoudar and Kim, 2000). Other routes include decarboxylation of *p*-hydroxybenzoate into phenol (Lupa et al., 2005, 2008), which appears interesting in terms of connecting the different upper funneling branches (see **5.4**) (Fig. 3).

5.1.3. S-type-lignin aromatics – sinapate, syringate, and syringaldehyde

Pathways required for the degradation of S-type-lignin associated aromatics are often underrepresented (Becker and Wittmann, 2019; Brink et al., 2019). Consequently, bacteria possessing these rare pathways are constantly under profound investigation, for example *Sphingobium* sp. strain SYK-6 (Araki et al., 2019, 2020; Uchendu et al., 2021), *P. putida* KT2440 (Nogales et al., 2011; Notonier et al., 2021), and lately, also, *N. aromaticivorans* (Cecil et al., 2018; Perez et al., 2019, 2021) (Table 1). Additionally, intense efforts are invested to find novel isolates possessing this substrate spectrum (Ravi et al., 2019; Shinoda et al., 2019).

Sinapate. Sinapate is found in different plants (Nićiforović and Abramovič, 2014). However, compared to broadly observed phenylpropanoids such as *p*-coumarate, sinapate is less prominent given its occurrence in only selected agricultural residues and at lower abundance (Karp et al., 2016; Sun et al., 2002). Despite a couple of reports about the bacterial metabolism of sinapate (Jurková and Wurst, 1993; Numata and Morisaki, 2015), the corresponding genes were largely not described. For *Sphingobium* sp. strain SYK-6, *in vitro* characterization of the ferulate degrading enzymes FerA and FerB showed, that sinapate was converted into syringaldehyde (Masai et al., 2002), and, additionally, sinapate was shown to serve as a substrate in actual growth experiments (Masai et al., 2004). Findings by (Kasai et al., 2012; Masai et al., 2002, 2004), thus, indicate, that this phenylpropanoid compound is degraded in analogy to ferulate *via* a CoA-dependent non-β-oxidation pathway (Fig. 3). The metabolic route for sinapate degradation *via* syringaldehyde and syringate was also affirmed in studies with *N. aromaticivorans* (Cecil et al., 2018). Lastly, corresponding to the previous phenylpropanoids, some marine bacteria were also reported to decarboxylate sinapate into 4-vinylsyringol (Ohta et al., 2012).

Syringaldehyde. The characterization of LigV from *Sphingobium* sp. strain SYK-6 suggested, that vanillin dehydrogenase is not solely responsible for syringaldehyde conversion (Masai et al., 2007b). This gap was recently filled by identifying DesV as the major syringaldehyde dehydrogenase (Kamimura et al., 2017a), and both enzymes were for example also predicted for the Gram-positive *Microbacterium* sp. strain RG1 capable of degrading S-lignin related compounds (Ravi et al., 2019).

Syringate. The further pathways involved in syringate conversion of *Sphingobium* sp. strain SYK-6 were thoroughly investigated in the last

two decades providing valuable insights into this microorganism's (S)-lignin related metabolism (Abe et al., 2005; Araki et al., 2019, 2020; Kasai et al., 2004; Masai et al., 2004). The tetrahydrofolate-dependent *O*-demethylase DesA catalyzes the *O*-demethylation of syringate into 3-*O*-methylgallate (Masai et al., 2004), which is then further sluiced *via* different routes and entry points into the protocatechuate-4,5-*meta*-cleavage pathway (Kasai et al., 2004) (see **5.3.3**) (Fig. 3).

5.1.4. C-type-lignin aromatics – caffeate

Caffeate. Interestingly, caffeate seemed never the sole center of attention but was rather invested as an add-on during the characterization of other phenylpropanoid degradation pathways. Also, several groups report about abiotic oxidation (Otani et al., 2014; Vardon et al., 2015), which appears limiting for experiments. This picture might change in the future, given its association with C-lignins (Vermaas et al., 2019), receiving emerging attention (Berstis et al., 2016), as well. So far, several studies provide evidence for the availability of the common bacterial strategies for side-chain shortening, e.g., the CoA-dependent non-β-oxidation (Meyer et al., 2018; Vardon et al., 2015), and CoA-dependent β-oxidation (Kallscheuer et al., 2016) pathways, and, also, the decarboxylation into 4-vinylcatechol (Cavin et al., 1998; Jung et al., 2013; L. Li et al., 2019a).

5.1.5. Lignin precursors and their derivatives - cinnamate and benzoate

Cinnamate. Cinnamate serves as a direct precursor for lignin biosynthesis (Rinaldi et al., 2016) and occurs as an intermediate during bacterial degradation of Kraft lignin (Raj et al., 2007; Shi et al., 2013). Notably, cinnamate lacks substituents at its aromatic ring, different to the previously mentioned phenylpropanoids, making this substrate even more recalcitrant and likely explaining the frequently observed lack of growth and enzymatic activity on this compound (Huang et al., 1994; Jung et al., 2013; Kallscheuer et al., 2016; Masai et al., 2002; Mpofu et al., 2019; Peng et al., 2003). Nevertheless, selected *Actinobacteria* can degrade cinnamate. In *Amycolatopsis* sp. strain ATCC 39116, degradation takes place *via* benzaldehyde and benzoate, leading finally to catechol (Sutherland et al., 1983), suggesting the existence of a CoA-dependent non-β-oxidation-pathway (Fig. 3). For *Rhodococcus opacus* PD630, a CoA-dependent β-oxidation-pathway was proposed, which consequently only results in benzoate (Cai et al., 2020), matching early observations for *Rhodococcus rhodochrous* (Andreoni et al., 1991). Notably, to our knowledge no bacterial decarboxylase (Cavin et al., 1998; Huang et al., 1994; Mpofu et al., 2019; Peng et al., 2003), has yet been found to accept cinnamate as a substrate, which, following the pattern for other decarboxylases, would result in the production of vinylbenzol (Fig. 3).

Benzoate. The capability to degrade benzoate is widespread among bacteria, and benzoate serves as a model compound in aromatic degradation studies since a long time (Harwood and Parales, 1996). As shown above, benzoate is also an intermediate during cinnamate breakdown (Fig. 3). Referring to the above-described degradation pathway in *P. putida* KT2440 (Fig. 2), benzoate is generally first converted into benzoate diol, whereby subsequent decarboxylation yields the central intermediate catechol (Jiménez et al., 2002; Park and Kim, 2003; Patrauchan et al., 2005; Shen et al., 2012).

5.2. Upper degradation routes for "technical" lignin aromatics

As stated above (see **2.2**), technical lignins differ massively in their structure from native lignins, and the necessary harsh methods required for its depolymerization (Rinaldi et al., 2016) often result in (alkylated) phenols and catechols (Fernández-Rodríguez et al., 2017; Kawamoto, 2017; Schutyser et al., 2018). Due to this, these aromatics are of high relevance, although they are not assigned to classical lignin degradation pathways (Fig. 3, Fig. S1, Supplementary File 1). Beneficially, different *Pseudomonas* strains have been well studied for their capability to degrade phenols, catechols, and cresols and their derivatives in the context of bioremediation (Díaz, 2004; van der Meer et al., 1992), which provides valuable knowledge to valorize lignin. In fact, making use of the genes for phenol degradation derived from *Pseudomonas* sp. strain CF600 (Powlowski and Shingler, 1994; Shingler et al., 1989), broadened the substrate spectrum of *P. putida* KT2440 for lignin-based applications (Kohlstedt et al., 2018; Vardon et al., 2015). Interestingly, the respective genes required for the metabolism of these aromatics often occur as mobile genetic elements, e.g., as plasmids, or, transposons, appearing suitable to ensure a fast adaption of microbial communities to an increased environmental occurrence of certain aromatics (Díaz, 2004; van der Meer et al., 1992), or pathway evolution (Díaz et al., 2013).

5.2.1. Degradation of toluate isomers, toluene, and xylene isomers

Next to benzoate, *P. putida* mt-2 was also described to grow on the monomethylated benzoate variants *p*-toluate (4-methylbenzoate), and *m*-toluate (3-methylbenzoate) (Murray et al., 1972). Only later, it was discovered, that this unspecific benzoate/toluate (methylbenzoate) metabolism is enabled by the catabolic TOL-plasmid (pWW0), which was curiously discovered independently by different researchers (Nakazawa, 2002; Nakazawa and Yokota, 1973; Williams and Murray, 1974; Wong and Dunn, 1974). The toluate-isomer metabolism proceeds analogous to that of benzoate, whereby the different substitutions lead to 3-methylcatechol and 4-methylcatechol as intermediates, respectively (Murray et al., 1972; Williams and Murray, 1974). The TOL plasmid, also, allows *P. putida* mt-2 to grow on toluene, *m*- and *p*-xylene (Worsey and Williams, 1975) (Fig. S1, Supplementary File 1), and exceptionally, the substrate spectrum of *N. aromaticivorans* covers even all xylene-isomers (Fredrickson et al., 1991) (Table 1).

5.2.2. Degradation of (alkylated) phenols

Phenol degradation proceeds *via* hydroxylation into catechol (Antai and Crawford, 1983; Szőköl et al., 2014; Xiao et al., 2015) **(Fig. 3)**. The multicomponent phenol monooxygenase from *Pseudomonas* sp. strain CF600 (Powlowski and Shingler, 1994; Shingler et al., 1989), was, additionally, shown to convert monomethylated phenols, i.e., different cresol isomers (Kohlstedt et al., 2018) (Fig. S1, Supplementary File 1). In the course of hydroxylation, both *m*- and *o*-cresol are converted into 3-methylcatechol, and *p*-cresol is transformed into 4-methylcatechol (Sala-Trepat et al., 1972). Approaching even higher alkylation, an alkylphenol hydroxylase was identified in *Rhodococcus* strains, catalyzing the hydroxylation of *p*-cresol (*p*-methylphenol), *p*-ethylphenol and even *p*-propylphenol, into their corresponding alkylcatechols (Levy-Booth et al., 2019). According to earlier studies, *p*- and *m*-cresol degradation additionally occurs *via* initial oxidation (Hewetson et al., 1978; Hopper and Taylor, 1975; Keat and Hopper, 1978a, 1978b), and the respective genes involved in *p*-cresol degradation were so-far characterized in different *Pseudomonas* strains (Chen et al., 2014; Cho, 2011; Jõesaar et al., 2010; Kim et al., 1994). Other works indicate an even broader variability of bacterial cresol-degradation pathways (Barton et al., 2018; Du et al., 2016; Kolomytseva et al., 2007), setting this group of aromatics somehow apart from the previously described.

5.3. Lower ring cleavage pathway routes around catechol and protocatechuate

5.3.1. The industrially most relevant β-ketoadipate pathway

The β-ketoadipate pathway is most important for microbial lignin valorization, as several of its intermediates are precursors to derive bio-based plastics, prominently *cis, cis*-muconate (Vardon et al., 2015) (7.1.1), but also, for instance, β-ketoadipate (Suzuki et al., 2021) (7.1.3). The β-ketoadipate pathway is probably the best-known route for aromatics conversion and seems reserved for soil microbes with a lifestyle, closely linked to plants (Harwood and Parales, 1996), and is apparently common in lignin-degrading bacteria (Granja-Travez et al., 2020). Essentially characterized more than 50 years ago, this pathway comprises two parallel branches, which are initiated by the *ortho*-cleavage of

catechol and protocatechuate, respectively (Ornston, 1966a; 1966b). The two ring-cleavage enzymes, catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase, have been thoroughly investigated (Harwood and Parales, 1996). Obviously, protocatechuate-3,4-dioxygenases exhibit high substrate specificity, whereas catechol-1,2-dioxygenases possess a broader substrate spectrum (Vetting and Ohlendorf, 2000). As an example, catechol-1,2-dioxygenase of *Amycolatopsis* sp. was shown to accept methylated catechols (An et al., 2001), a property, which was later useful to derive methylated *cis, cis*-muconate variants (Barton et al., 2018). Downstream, the two branches of the β-ketoadipate pathway re-merge after analogous reactions at β-ketoadipate enol lactone, which is channeled *via* the central β-ketoadipate pathway towards the TCA cycle metabolites acetyl-CoA and succinyl-CoA (Harwood and Parales, 1996) (Fig. 4).

5.3.2. 2,3-Meta cleavage of catechol and protocatechuate

The function of the catechol-2,3-*meta* cleavage pathway lies within the catabolism of catechol and methylcatechols, obtained from toluates (Murray et al., 1972), toluene and xylenes (Worsey and Williams, 1975), and cresols (Sala-Trepat et al., 1972). Interestingly, ring-cleavage of different intermediates is catalyzed by a single nonspecific catechol-2,3-dioxygenase, whereby further catabolism of the cleavage products is solved by two divergent branches, namely the oxidative branch/4-oxalocrotonate branch and the hydrolytic branch (Murray et al., 1972; Sala-Trepat et al., 1972) (Fig. S1, Supplementary File 1). Even though, structurally both routes appear possible for catechol, it is assumed, that the oxidative branch is preferred (Murray et al., 1972; Sala-Trepat et al., 1972). The metabolism of alkylated catechols in *Rhodococcus* is basically identical to the oxidative branch under consideration of the varying side chains (Levy-Booth et al., 2019), and also serves for the metabolism of alkylated guaiacols (Fetherolf et al., 2020). Curiously, also the protocatechuate-2,3-*meta* cleavage pathway, first described in the late 1970s (Crawford, 1975; Crawford et al., 1979), shares striking similarities with this catechol degradation route and yields pyruvate and acetyl-CoA as final metabolites (Kasai et al., 2009). The responsible *pra*-gene cluster was uncovered in *Paenibacillus* sp. strain JJ-1b (Kasai et al., 2009). Interestingly, the metabolic similarities between the pathways encoded by the *pra* (protocatechuate 2,3-*meta* cleavage)- and *xyl* (catechol 2,3-*meta* cleavage)-gene clusters (Fig. 4) match observations for *N. aromaticivorans*, where *xyl*-homologues were found to be involved in protocatechuate degradation (Cecil et al., 2018).

5.3.3. The protocatechuate-4,5-meta-cleavage pathway for S-lignin aromatics

Degradation of protocatechuate *via* the 4,5-*meta* cleavage pathway mainly occurs in α- and β-*Proteobacteria* (Ni et al., 2013), and was characterized in several bacteria (Kamimura et al., 2010; Maruyama et al., 2004; Ni et al., 2013; Providenti et al., 2001). For lignin valorization, the *lig*-gene cluster of *Sphingobium* sp. strain SYK-6 (Hara et al., 2000, 2003; Masai et al., 1999, 2000; Noda et al., 1990) and the corresponding genes in *N. aromaticivorans* (Cecil et al., 2018) seem most relevant (Table 1). Ring cleavage by 4,5-protocatechuate dioxygenase LigAB yields 4-carboxy-2-hydroxymuconate-6-semialdehyde (Noda et al., 1990), which is further converted into 2-pyrone-4,6-dicarboxylate by LigC (Masai et al., 2000) and 4-oxalomesaconate by LigI (Masai et al., 1999). Only recently, it was described that 4-oxalomesaconate is isomerized into 2-keto-4-carboxy-3-hexenedioate by a 1,3-allylic isomerase LigU (Hogancamp and Raushel, 2018), followed by hydration into 4-carboxy-4-hydroxy-2-oxoadipate (LigJ) (Hara et al., 2000). Subsequent aldolytic cleavage (LigK) yields pyruvate and oxaloacetate, which is, thereupon, decarboxylated into pyruvate (Hara et al., 2003). The degradation of S-lignin related aromatics *via* 3-*O*-methylgallate and gallate is closely connected to the protocatechuate-4,5-*meta* cleavage pathway and this metabolic node was extensively characterized in *Sphingobium* sp. strain SYK-6 (Araki et al., 2020; Kasai et al., 2004, 2005) (Fig. 4), and only lately for *N. aromaticivorans* (Perez et al., 2021). Notably, a gallate ring-cleavage pathway was also described for *P. putida* KT2440 (Nogales et al., 2005, 2011). Even though for *P. putida* KT2440, initially a tautomerization of 4-oxalomesaconate keto-form into its enol-form was suggested (GalD) (Nogales et al., 2011), a 1,3-allylic isomerization into 2-keto-4-carboxy-3-hexenedioate appears feasible, based on the characterization of its homologue LigU in *Sphingobium* sp. strain SYK-6 (Hogancamp and Raushel, 2018). From there, this pathway encoded by the *gal*-gene cluster (Nogales et al., 2005, 2011) follows the same conversions as described for the protocatechuate-4,5-*meta* cleavage pathway (Fig. 4).

5.4. Coupling of funneling branches by decarboxylases

Decarboxylases add a substantial layer of metabolic versatility by enabling an interconnection of different upper pathways (Fig. 3, Fig. S1, Supplementary File 1, **green arrows**), suggesting that the commonly strict linear categorization of the upper pathways not fully captures the highly flexible metabolic network in nature (Crawford and Olson, 1978). The involved co-factor independent hydroxyarylic acid decarboxylases/phenol carboxylases form a new enzyme family (Lupa et al., 2005, 2008). For example, *Amycolatopsis* sp. strain ATCC 39116 either demethylates vanillate into protocatechuate or decarboxylates it into guaiacol, which is subsequently demethylated into catechol (Meyer et al., 2017; Pometto III et al., 1981; Sutherland et al., 1983). The decarboxylation pathway, enabled by vanillate decarboxylase, even displays the major metabolic route (Meyer et al., 2017). However, microbes not always convert the decarboxylation products (Chow et al., 1999; Crawford and Olson, 1978; Graf et al., 2016), giving opportunity for shared substrate utilization in microbial communities (Chow et al., 1999; Lupa et al., 2008). From a biotechnology viewpoint, the “crosstalk” enabled by certain decarboxylases (Chow et al., 1999), is of immense value, as impressively demonstrated for *cis, cis*-muconate production (Vardon et al., 2015).

6. Metabolic engineering strategies

6.1. The mining for novel microbial hosts for the lignin industry - beneficial built-in properties

Over the years, a selection of different bacteria has emerged to be of significant value for lignin valorization (Bugg et al., 2021). Among the well-performing hosts, there seems no single optimal one, but the different strains are rather complementary in terms of robustness, growth speed and genetic accessibility, each providing individual advantages (and drawbacks) for a given substrate stream and product of interest (Becker and Wittmann, 2019; Beckham et al., 2016). Also, the capability of bacterial hosts to actually, depolymerize lignin is frequently pointed out to be of advantage for lignin-valorization concepts (Salvachúa et al., 2015). As an interesting side-effect and because not all bacterial hosts have been intensively studied before, strategies for an upgrading of lignin or associated aromatic compounds are also drivers for the development of e.g., genetic engineering tools (Barton et al., 2018; Cai et al., 2020; Fleige and Steinbüchel, 2014; Meyer et al., 2017).

Although the available strains have revealed a remarkable potential (Table 1), it is tempting to assume that an even better host might just wait around the corner, explaining the on-going search for novel isolates with eventually superior lignin degradation properties and novel pathways for aromatic assimilation (Fig. 1). Here, nature offers various possibilities (Brink et al., 2019). For instance, warm (Levy-Booth et al., 2021; Peng et al., 2003) and cold (Margesin et al., 2021) isolation sites allow to discover enzymes, specifically tailored for a certain temperature profile of lignin valorization. Considering the natural occurrence of lignocellulosic biomass and plant matter, the majority of bacteria involved in lignin mineralization is hereby derived from soil, e.g., of Alpine forests (Margesin et al., 2021), hot springs (Crawford et al.,

1979), agriculturally-used areas (Moraes et al., 2018). In addition, samples of plant origin are intriguing (Brink et al., 2019), just thinking of the powerful Kraft-lignin degraders, gained from leaf mold samples (Xu et al., 2018) and the steeping fluid of eroding bamboo slips (Shi et al., 2013, 2017). Moreover, rooting in the symbiosis of bacteria and animals, prominent microbes may also be isolated from termite gut (Zhou et al., 2017) and ruminal fluids (Degrassi et al., 1995).

Less obvious, also marine bacteria appear to be valuable, which can be attributed to terrestrial plant matter transported into the sea by rivers (Lu et al., 2020), sunken wood (Ohta et al., 2012), and curiously, might also be related to reports of lignin in seaweed (Alzate-Gaviria et al., 2020; Martone et al., 2009). As shown for *Oceanimonas doudoroffi* (Numata and Morisaki, 2015), lignin depolymerization could be a more general feature of marine bacteria, considering a lessened activity of wood-decaying fungi in marine environments, and a sensitivity of some lignin-converting enzymes to saline conditions (Ohta et al., 2012). Also, *Cyanobacteria* might play a certain role in lignin degradation (Bharati et al., 1992), substantiated by *in silico*-predicted lignin degrading enzymes or aromatic degradation pathways (Brown et al., 2012; Bugg et al., 2011a; Tian et al., 2014). Beyond nature, artificial aromatic-rich environments generated by mankind are prominent sources for lignin/aromatic degraders (Brink et al., 2019), including the effluents of pulp and paper mills (Haq et al., 2016; Mathews et al., 2016), xenobiotics-polluted land (Seto et al., 1995) and water (Jõesaar et al., 2010; Numata and Morisaki, 2015).

6.2. Upgrading of performance - creation of tailor-made synthetic properties

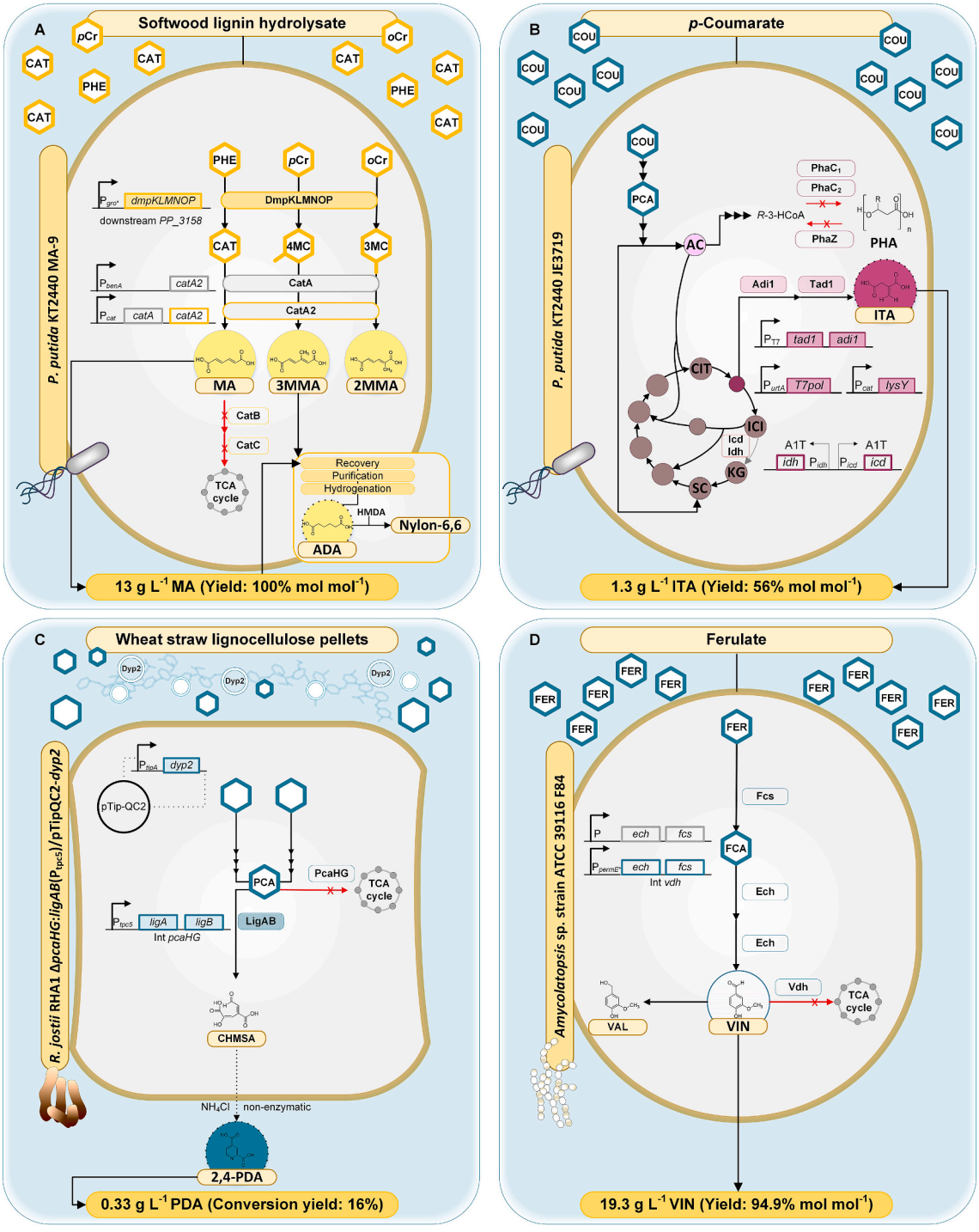
Generally, a microbial producer is evaluated based on key performance indicators such as titer, yield, and productivity. These are also important parameters, when striving for lignin valorization, and typically require a substantial upgrading of the biosynthetic potential.

Broadening of the substrate spectrum. Depending on the lignin type, and the depolymerization method, different mixtures of structurally variable aromatic monomers are obtained (Schutyser et al., 2018). Especially, the aromatics arising from harsh depolymerization methods (Fernández-Rodríguez et al., 2017; Kawamoto, 2017; Schutyser et al., 2018), which partially have been once in the focus of bioremediation (van der Meer et al., 1992), are likely not all covered by the natural aromatic substrate spectrum of a single bacterium. Additionally, recent studies suggest that naturally, microbial populations apply a "task division strategy", where an efficient and wholesome lignin catabolism relies on complementary and interlocking metabolic pathways (Díaz-García et al., 2020), which is also in line with "dead-end" metabolites (Chow et al., 1999; Lupa et al., 2008). The broadening of the aromatic substrate spectrum appears, therefore, necessary to create efficient single-strain cell factories for the upgrading of lignin waste streams, and various studies have come up with different solutions. For example, guaiacol utilization was identified as a key target for efficient lignin valorization (Beckham et al., 2016), stimulating studies focusing on its assimilation in natural degraders (Barton et al., 2018; Mallinson et al., 2018), and the transfer of the desired guaiacol degradation capabilities into the lignin-relevant host *P. putida* (García-Hidalgo et al., 2019; Tumen-Velasquez et al., 2018). Likewise, heterologous expression of the multicomponent phenol hydroxylase from *Pseudomonas* sp. strain CF600 in *P. putida* KT2440 allowed the aspired degradation of phenol and, also, different cresols (Fig. 5A), enabling the production of methylated *cis, cis*-muconate variants towards polymers with novel properties (Kohlstedt et al., 2018). The realization of metabolic pathways for other substrates even require novel protein functions, as demonstrated for the S-lignin relevant aromatic syringol (Machovina et al., 2019). Because of a lack of natural pathways, its valorization requires approaches beyond heterologous gene expression. By substitution of the amino acid F169 in the active site of guaiacol-*O*-demethylase P450 oxidase subunit GcoA, an altered enzyme specificity towards syringol was achieved, likewise keeping the natural activity towards guaiacol (Machovina et al., 2019). Heterologous expression of the GcoA-F169A-GcoB variant in a *P. putida* KT2440-derivative, allowed a sequential demethylation of syringol into pyrogallol, however a profound further metabolism still must be established (Machovina et al., 2019). Worth mentioning at this point are bacterial co-cultures that distribute the metabolic tasks between different microbes and, this way, approach the natural cooperation in consortia (He et al., 2017).

Enhancing aromatics uptake. Metabolic engineering of transport is *per se* an acknowledged target to improve production performance (Kind et al., 2011). Admittedly, aromatics are toxic (Becker and Wittmann, 2019), so that overexpressing responsible transport proteins to increase the uptake of these molecules might seem counterproductive on first glance. However, metabolic engineering of aromatics import can indeed have a positive influence on production performance (Mori et al., 2018). For example, the overexpression of the protocatechuate transporter gene *pcaK* in *Sphingobium* sp. strain SYK-6, enabled an almost 30% higher conversion into the plastics-precursor 2-pyrone-4,6-dicarboxylate (Mori et al., 2018). Clearly, this field is not well-developed today. Even though selected proteins involved in aromatics uptake were reported (Kamimura et al., 2017b), we still lack knowledge on their properties and impact in different hosts (Nichols and Harwood, 1997; Wada et al., 2021), so that more research is needed.

Deregulating native catabolic pathways from undesired control. Regulators play a major role for the host cells' substrate spectrum (Powlowski and Shingler, 1994), pointing out the need to understand the native metabolism of bacterial chassis strains to fully profit from their metabolic network. For instance, *P. putida* KT2440 was, astonishingly, shown to convert syringate in co-presence of vanillate, suggesting the involvement of a native vanillate-*O*-demethylase VanAB (Sonoki et al., 2018). Indeed, a *vanAB* deletion mutant lost the capability to utilize syringate in presence of an auxiliary energy source and growth on syringate as sole carbon and energy source required the integration of a second copy of *vanAB* under control of the strong *tac* promoter (Notonier et al., 2021). The impact of circumventing local regulatory mechanisms becomes also obvious from the fact that only a mutant of *C. glutamicum* that lacked the repressor PhdR, involved in phenylpropanoid metabolism, could grow on cinnamate as the sole carbon and energy source (Kallscheuer et al., 2016) (Fig. 3). In addition, natural regulation might become a problem, regarding the common substrate mixtures in lignin hydrolysates (Johnson et al., 2017). In *P. putida* KT2440 phenylpropanoid assimilation is limited by carbon catabolite repression, and also, accumulated aromatic metabolic intermediates stand in competition with high product yields (Johnson et al., 2017; Vardon et al., 2015). Notably, some works have already aimed at a circumvention of native regulatory mechanisms by metabolic engineering, either on a local basis by a direct synthetic control *via* promoter exchange in *C. glutamicum* (Becker et al., 2018), or on a more global basis by deleting the CRC global regulator in *P. putida* KT2440 (Johnson et al., 2017; Niu et al., 2020). Of course, substrate preferences must be evaluated for each bacterial host, considering that *Amycolatopsis* sp. strain ATCC 39116 prefers aromatics over glucose (Barton et al., 2018), and *Sphingobium* sp. strain SYK-6 grows actually very poor on sugars, as its physiology is fully aligned to lignin catabolic pathways (Varman et al., 2016).

Improving tolerance. Aromatic compounds are toxic (Becker and Wittmann, 2019), and bacteria have therefore evolved defense mechanisms including an adaption of the cell membrane (Haußmann and Poetsch, 2012), a controlled expression of transporters (Yoneda et al., 2016), metabolic detoxification (Brink et al., 2019), and enzymatic "safety valves" (Jiménez et al., 2014). In presence of toxic substrates cells compromise between productivity and persistence (Gong et al., 2017), highlighting, why tolerance engineering is undeniably crucial for the use of aromatics (Mohamed et al., 2020). In particular, catechol toxicity displays a huge issue for lignin valorization (Cai et al., 2020; Kaneko et al., 2011; Kohlstedt et al., 2018). Surprisingly, overexpression of the major catechol-1,2-dioxygenase *catA* in *P. putida* KT2440 by



(caption on next page)

Fig. 5. Systems metabolic engineering of different bacterial hosts for the valorization of lignin and lignin-derived aromatic monomers. (A) Deletions of *catB* (*cis, cis*-muconate cycloisomerase), *catC* (muconolactone isomerase), *endA-1, endA-2* (endonucleases (not shown)), overexpression of *catA2* (catechol-1,2-dioxygenase) by second gene copy under control of P_{cat}-promoter, introduction of heterologous *dmpKLMNOP*-genes (phenol hydroxylase) from *Pseudomonas* sp. strain CF600 under control of P_{gro*}-promoter (Kohlstedt et al., 2018), **(B)** Deletions of $phaC_1$, $phaC_2$ (polyhydroxyalkanoate synthetases), *phaZ* (polyhydroxyalkanoate depolymerase), decrease in translation efficiency of divergently transcribed and adjacent *icd-*, *idh*-genes (isocitrate dehydrogenase) (thin line) by exchanging the start codons from ATG to TTG, introduction of heterologous *adi1* (aconitate isomerase), *tad1* (*trans*-aconitate decarboxylase) from *Ustilago maydis* under control of T7 promoter, dynamic regulatory system: T7*pol* (T7 polymerase) under control of nitrogen-sensitive promoter P_{urtA}, *lysY* (catalytically-deactivated variant of T7 lysozyme, allosteric inhibitor of T7 polymerase) constitutively expressed under P_{cat} (pLysS) (Elmore et al., 2021), **(C)** Introduction of heterologous *ligAB* (protocatechuate-4,5-dioxygenase) from *Sphingobium* sp. strain SYK-6 under control of the constitutive promoter P_{tpc5} in place of the native *pcaHG*-genes (protocatechuate-3,4-dioxygenase), pTip-QC2-based expression of heterologous *dyp2* (lignin oxidizing enzyme) from *Amycolatopsis* sp. strain ATCC 39116 (Spence et al., 2021), **(D)** Deletion of *vdh* (vanillin dehydrogenase), second gene copies of *ech* (enoyl-CoA hydratase/lyase) and *fcs* (feruloyl-CoA synthetase) under control of the constitutive promoter *permE** into the *vdh*-gene locus (Fleige et al., 2016). Metabolite abbreviations: AC – Acetyl-CoA, ADA – Adipate, CAT – Catechol, PHE – Phenol, CHMSA – 4-Carboxy-2-hydroxymuconic-6-semialdehyde, CIT – Citrate, oCr – *o*-Cresol, pCr – *p*-Cresol, COU – *p*-Coumarate, FER – Ferulate, FCA – Feruloyl-CoA, HMDA – Hexamethylene diamine, *R*-3-HCoA – (*R*)-3-Hydroxacyl-CoA, ICI – Isocitrate, ITA – Itaconate, KG – α-Ketoglutarate, MA – *cis,cis*-Muconate, 3-MC – 3-Methylcatechol, 4-MC – 4-Methylcatechol, 2-MMA – 2-Methyl-*cis,cis*-muconate, 3-MMA – 3-Methyl-*cis,cis*-muconate, PCA – Protocatechuate, PHA – Polyhydroxyalkanoates, 2,4-PDCA – Pyridine-2,4-dicarboxylate, SC – Succinyl-CoA, VIN – Vanillin, VAL – Vanillyl alcohol. Symbols: genes – angular squares, catabolic proteins – rounded squares, lignin depolymerizing enzymes – concentric circles, aromatics – hexagons. Arrows: black – metabolic transformation, red, crossed – gene deletion and loss of respective gene function, dashed black – enzymatically independent reaction. Colors: yellow – engineered catechol/methylcatechol associated pathways, blue – engineered protocatechuate associated pathways, pink – engineered tricarboxylic acid cycle associated pathways, grey – genes/proteins unaffected by genetic modifications; colored, filled-out boxes – heterologous genes/proteins, outlined boxes – native genes/proteins.

strong synthetic promoters did not lead to improved catechol conversion, but mimicking natural tolerance mechanisms proved to be far more effective (Kohlstedt et al., 2018). The integration of a second genomic copy of the *catA2* gene downstream of *catA* under control of the native P_{cat} promoter, enabled a remarkable 20% improvement in catechol-tolerance (Kohlstedt et al., 2018). Genetic engineering of separate metabolic features might, however, fizzle up in view of the complexity of cellular responses, which contribute to cell robustness (Becker and Wittmann, 2019; Gong et al., 2017). Therefore, combinatory approaches of adaptive laboratory evolution, genome sequencing and reverse genetic engineering might add value (Becker and Wittmann, 2019; Gong et al., 2017), as recently shown for the tolerance of *P. putida* KT2440 against *p*-coumarate and ferulate (Mohamed et al., 2020), and also other studies focusing on aromatic mixtures (Henson et al., 2018) and corn stover hydrolysates (Wang et al., 2018).

6.3. Advances in systems biology and in silico simulation

Equally in novel isolates (García-Hidalgo et al., 2020; Ravi et al., 2018; Shinoda et al., 2019) and known hosts (Notonier et al., 2021), a profound systems level characterization of the metabolic potential is crucial for applications in lignin valorization (Fig. 1). Transcriptomics were particularly useful to identify novel aromatic pathways in lab grown microbes (Henson et al., 2018; Kallscheuer et al., 2016; Levy-Booth et al., 2019), and also proteomics (X. Li et al., 2019b; Meyer et al., 2018), and fluxomics (Roell et al., 2019; Varman et al., 2016) have added important knowledge. Next, metagenomics and metatranscriptomics provided valuable insights into microbial communities (Díaz-García et al., 2020; Levy-Booth et al., 2021; Moraes et al., 2018). Complementary, *in silico* tools have become indispensable for getting holistic views on lignin degrading and assimilating bacteria (Gonçalves et al., 2020; Tian et al., 2014), and have proven as useful to identify novel ligninolytic enzymes (Ahmad et al., 2011; Brown et al., 2012; Brown and Chang, 2014; Tian et al., 2014; Xu et al., 2021), and enzyme variants for aromatic assimilation (García-Hidalgo et al., 2019; Jung et al., 2013). A very interesting approach, recently shown, seeks for potential pathways on basis of enzyme promiscuity and computationally prospects possible ways of funneling lignin-derived mono- and biaryls under given reaction rules and within mass balanced metabolic networks (Wang and Maranas, 2021). This modelling framework appears of great help considering the still limited experimental knowledge. In addition, digital bibliographies and databases have emerged as valuable resource for metabolic engineers (Gonçalves et al., 2020; Tian et al., 2014). The *eLignin Microbial Database* provides structural insights on microorganisms, pathways and substrates associated with this topic (Brink et al., 2019).

7. Industrial showcases for lignin valorization

From the metabolic engineering viewpoint, there are basically two success strategies to harness the metabolism of aromatic compounds (Becker and Wittmann, 2019). Biotransformations directly transform aromatics to industrially valuable metabolites, especially bioplastic precursors, e.g., *cis, cis*-muconate (Becker et al., 2018; Cai et al., 2020). This strategy mostly implies a disruption of the aromatic pathways downstream of the target product, the use of a second substrate for growth and supply of energy and redox power, plus a range of supporting modifications to increase performance (see 7.1–7.2). *De novo* synthesis from aromatics, e.g., polyhydroxyalkanoate production (Borrero-de Acuña et al., 2020; Salvachúa et al., 2020a), builds on the close interweavement of the aromatic metabolism and the TCA cycle (Fig. 4) to produce metabolites starting from central carbon metabolism (see 7.3–7.6) (Table 2, Table S1, Supplementary File 2).

7.1. Precursors for biomaterials

For biobased industries, including the lignin industry, bulk chemicals, including plastic precursors, display a unique opportunity (Tuck et al., 2012). These valorization routes are also interesting in terms of alleviating environmental concerns around the conventional (petro) chemical production routes of some of these chemicals, e.g., *cis, cis*-muconate (Khalil et al., 2020), adipate (Corona et al., 2018) and styrene-derivatives (Chen et al., 2018; Mishra et al., 2014). The lower ring cleavage pathways of aromatic catabolism (Fig. 4) generate several dicarboxylic acids as metabolic intermediates, providing a solid foundation for high-yield production of different bioplastic precursors (Johnson et al., 2019).

7.1.1. The flagship of lignin valorization – cis, cis-muconate

Muconate is a di-unsaturated dicarboxylic acid which strikingly functions as a central precursor for the production of different commercially relevant bulk chemicals, including, among others, adipate and terephthalate (Khalil et al., 2020; Xie et al., 2014). Additionally, muconate is also interesting as a polymer building block itself (Khalil et al., 2020). This versatility substantiates its labelling as a "bio-privileged" molecule (Shanks and Keeling, 2017), as well as its related high global market potential, that was estimated to exceed $ 22 billion per year (Sonoki et al., 2018). Besides *de novo* synthesis of *cis, cis*-muconate from glucose, as innovatively shown by Draths and Frost (1994), the molecule is, naturally, produced as a metabolic intermediate in the β-ketoadipate pathway (Fig. 4). The first *cis, cis*-muconate producer, characterized on genetic level, was *P. putida* KT2440-JD1, containing a point mutation in the *catR*-gene, which encodes for the regulator CatR

Table 2

Showcases for the bacterial upgrading of lignin and lignin-model compounds into value-added products. [a]: inferred from reference; [b]: given as wt/wt_{cdw}. For the products, the following abbreviations were used: adipate (ADA), carotenoids (CAR), *cis, cis*-muconate (*cis, cis*-MA), alkylated *cis, cis*-muconate variants (2-CH_3-MA, 3-CH_3-MA), itaconate (ITA), *p*-hydroxybenzaldehyde (*p*-HBA), β-ketoadipate (β-KA), lactate (LAC), polyhydroxyalkanoates (PHA), pyridine-2,4-dicarboxylate (2,4-PDA), pyridine-2,5-dicarboxylate (2,5-PDA), pyruvate (PYR), 2-pyrone-4,6-dicarboxylate (PDC), vanillin (VIN), 4-vinylguaiacol (4-VG), 4-vinylphenol (4-VP). A full overview on previous studies for successful upgrading of lignin into chemicals is given in the supplement (Table S1, Supplementary File 2).

Bacterium	Relevant features/Designation	Substrate	Product	Method	Titer [g L^{-1}]	Yield [mol mol^{-1}], [%]	Reference
P. putida KT2440	AA-1	*p*-Hydroxybenzoate	ADA	Fed-batch	2.5	17.4	Niu et al. (2020)
R. rhodochrous	Wildtype	Benzoate	CAR	Batch	0.0018[a]	–	Chen and Wan (2021)
P. putida KT2440	CJ238	*p*-Coumarate	*cis, cis*-MA	Batch	2.84[a]	94.6	Johnson et al. (2017)
R. opacus PD630	MA-6	Corn-stover lignin	*cis, cis*-MA	Fed batch	1.63	–	Cai et al. (2020)
Amycolatopsis sp. strain ATCC 39116	MA-2	Guaiacol	*cis, cis*-MA	Fed batch	3.10	96.0	Barton et al. (2018)
C. glutamicum	MA-2	Catechol	*cis, cis*-MA	Fed-Batch	85.0	100.0	Becker et al. (2018)
C. glutamicum	MA-2	Softwood lignin hydrolysate	*cis, cis*-MA	Fed batch	1.80	100.0	Becker et al. (2018)
P. putida KT2440	MA-1	Catechol	*cis, cis*-MA	Fed-batch	64.2	100.0	Kohlstedt et al. (2018)
P. putida KT2440	MA-9	Softwood lignin hydrolysate	*cis, cis*-MA 3-CH_3-MA	Fed-batch	13.0	100.0	Kohlstedt et al. (2018)
P. putida KT2440	CJ103	Alkaline pre-treated liquor	*cis, cis*-MA	Batch	0.70	67.0	Vardon et al. (2015)
P. putida KT2440	JD1	Benzoate	*cis, cis*-MA	Fed-batch	18.5	100.0	van Duuren et al. (2012)
E. coli BL21 (DE3)	*catA* (*P. putida* mt-2)	Catechol	*cis, cis*-MA	Fed-batch	59.0	100.0	Kaneko et al. (2011)
Sphingobium sp. strain SYK-6	SME257/pTS084	Birch lignin hydrolysate	*cis, cis*-MA	Fed-Batch	0.03[a]	41.2[a]	Sonoki et al. (2018)
Pseudomonas sp. strain NGC703	pTS084	Vanillate	*cis, cis*-MA	Fed-batch	3.2	75.0	Shinoda et al. (2019)
Pseudomonas sp. strain NGC703	pTS084	Birch derived phenols	*cis, cis*-MA	Fed-batch	0.14	–	Shinoda et al. (2019)
P. putida KT2440	CJ242	Benzoate	*cis, cis*-MA	Fed-batch	52.3	97.0	Salvachúa et al. (2018)
P. putida KT2440	JE3719	*p*-Coumarate	ITA	Batch	1.26	56.0	Elmore et al. (2021)
P. putida KT2440	dJ/pKAD	Protocatechuate	β-KA	Fed-batch	16.0[a]	100.0[a]	Okamura-Abe et al. (2016)
P. putida KT2440	dJ/pJBV-pKAD	Vanillin	β-KA	Fed-batch	24.70	93.9	Suzuki et al. (2021)
P. putida KT2440	CJ263	*p*-Hydroxybenzoate	β-KA	Fed-batch	41.1	107.8	Johnson et al. (2019)
R. opacus PD630	Wildtype	Corn stover lignin stream	Lipids	Fed-batch	1.83	18.1[a,b]	Liu et al. (2018)
R. opacus PD630		Alkali corn stover lignin	Lipids	Batch	–	33.0[b]	He et al. (2017)
R. jostii RHA1	VanA$^-$						
R. opacus PD630	Wildtype	*p*-Hydroxybenzoate	Lipids	Batch	–	20.3[b]	Kosa and Ragauskas (2012)
R. opacus PD630	Wildtype	Kraft lignin	Lipids	Batch	0.15[a]	–	Zhao et al. (2016)
R. jostii RHA1	WT:pTip-*atf8*	Benzoate	Lipids	Batch	–	65.0[a,b]	Amara et al. (2016)
P. putida KT2440	AG2162	*p*-Coumarate	PHA	Fed-Batch	0.95	54.2[b]	Salvachúa et al. (2020a)
P. putida KT2440	AG2162	Lignin containing stream (corn stover)	PHA	Batch	0.12	17.7[b]	Salvachúa et al. (2020a)
P. putida H	Δ*catA2*	Benzoate	PHA	Fed-batch	6.10	28.9 [a,b]	Borrero-de Acuña et al. (2020)
P. putida H	Δ*catA2*	Kraft lignin hydrolysate	PHA	Fed-batch	1.40	25.9 [a,b]	Borrero-de Acuña et al. (2020)
Pandoraea sp.strain B-6	Wildtype	Kraft lignin	PHA	Batch	0.17[a]	24.7	Liu et al. (2019)
Pandoraea sp. strain ISTKB	Wildtype	*p*-Hydroxybenzoate	PHA	Batch	0.41	60.0[b]	Kumar et al. (2017)
P. putida KT2440	Wildtype	Alkaline pretreated liquor	PHA	Batch	0.25	32.0[b]	Linger et al. (2014)
O. doudoroffi	Wildtype	Lignin	PHA	Batch	–	0.20[b]	Numata and Morisaki (2015)
P. putida NX-1	Wildtype	Kraft lignin	PHA	Batch	0.11	37.6[b]	Xu et al. (2021)
R. jostii RHA1	pTipQC2-*ligAB*	Wheat straw lignocellulose	2,4-PDA	Batch	0.13	–	Mycroft et al. (2015)
R. jostii RHA1	Δ*pcaHG*:*ligAB*(P_{tpc5}) pTipQC2-*dyp2*	Wheat straw lignocellulose	2,4-PDA	Batch	0.33	–	Spence et al. (2021)
R. jostii RHA1	pTipQC2-*praA*	Wheat straw lignocellulose	2,5-PDA	Batch	0.11	–	Mycroft et al. (2015)
R. jostii RHA1	Δ*pcaHG*/pTipQC2-*praA*	Wheat straw lignocellulose	2,5-PDA	Batch	0.29	–	Spence et al. (2021)
P. putida KT2440	CJ122	*p*-Coumarate		Batch			

(*continued on next page*)

Table 2 (*continued*)

Bacterium	Relevant features/Designation	Substrate	Product	Method	Titer [g L^{-1}]	Yield [mol mol^{-1}], [%]	Reference
			PYR		1.4[a]	65.8[a]	Johnson and Beckham (2015)
			LAC		1.4[a]	65.0[a]	
P. putida PpY1100	pDVABC	Protocatechuate	PDC	Fed-batch	11.8[a]	19.8[a]	Otsuka et al. (2006)
P. putida KT2440	AW045	Syringate, *p*-coumarate, and ferulate mixture	PDC	Batch	0.79[a]	93.0	Notonier et al. (2021)
N. aromaticivorans DSM 12444	Δ*ligI* Δ*desCD*	Depolymerized poplar lignin	PDC	Batch	0.09[a]	59.0	Perez et al. (2019)
N. aromaticivorans DSM 12444	Δ*ligI* Δ*desCD*	Vanillate Vanillin	PDC	Fed-batch	4.9	–	Perez et al. (2019)
P. putida KT2440	CJ251	*p*-Hydroxybenzoate	PDC	Fed-batch	58.0	80.7	Johnson et al. (2019)
Streptomyces sp. strain V-1	Wildtype	Ferulate	VIN	Fed-batch	19.2	54.5	Hua et al. (2007)
Amycolatopsis sp. strain ATCC 39116	Δ*vdh* (Strain F33)	Ferulate	VIN	Fed-batch	14.4	80.9	Fleige et al. (2016)
Amycolatopsis sp. strain ATCC 39116	Δ*vdh*::*permE**::*ech-fcs* (strain F84)	Ferulate	VIN	Fed-batch	19.3	94.9	Fleige et al. (2016)
P. putida KT2440	GN442	Ferulate	VIN	Batch	1.31[a]	86.0[a]	Graf and Altenbuchner (2014)
R. jostii	RHA045 (Δ*vdh*)	Wheat straw lignocellulose	VIN *p*-HBA	Batch	0.10 0.05–0.06	–	Sainsbury et al. (2013)
E. coli BL21 (DE3)	Phenolic acid decarbox. *B. atrophaeus*	Ferulate	4-VG	Fed-batch	237.3	98.9	Li et al. (2019a)
Amycolatopsis sp. strain ATCC 39116	Wildtype	Ferulate	4-VG	Batch	0.89	111.0	Max et al. (2012)
B. pumilus	Wildtype	Ferulate	4-VG	Fed-batch	7.6	80.0	Lee et al. (1998)
E. coli Rosetta (DE3)	Phenolic acid decarbox. *B. licheniformis*	Ferulate	4-VG	Fed-batch	129.9	85.6	Chen et al. (2018)
P. putida KT2440	*Δech::padC (B. subtilis)*	Green Value Protobind lignin	4-VG	Batch	0.06	–	Williamson et al. (2020)
E. coli BL21 (DE3)	Phenolic acid decarbox. *B. amyloliquefaciens*	*p*-Coumarate	4-VP	Batch	31.9	88.7	Jung et al. (2013)

(van Duuren et al., 2011). The high production rate in comparison to other *cis, cis*-muconate producers at that timepoint (van Duuren et al., 2011), can be seen as a starting shot, for the distinctive application of *P. putida* KT2440 for the production of this valuable compound (Table S1, Supplementary File 2).

Due to the natural setup of aromatic degradation, only compounds which are metabolized *via* the catechol-branch are converted into *cis, cis*-muconate (Fig. 4). Installing the protocatechuate decarboxylase AroY from *Enterobacter cloacae* in *P. putida* KT2440 in a pioneering approach, cross-connected naturally separated funneling pathways at the level of their central intermediates (see above) and enabled the channeling of compounds, metabolized *via* protocatechuate, towards the catechol branch (Vardon et al., 2015). Additionally, *P. putida* KT2440-CJ103 was equipped with a phenol hydroxylase encoded by *dmpKLMNOP* from *Pseudomonas* sp. strain CF600 for phenol utilization, a deletion of the *catBC*-genes to eliminate *cis, cis*-muconate-degradation, a decoupling of *catA*-expression from natural control by a promoter exchange (P_{tac}), and a deletion of the regulator encoding *catR*-gene. The mutant produced *cis, cis*-muconate from a range of substrates with yields spanning from 14% (coniferyl alcohol) to 93% (benzoate) (Vardon et al., 2015). This engineering strategy was subsequently fine-tuned by co-expression of the required helper proteins of AroY, namely EcdBD (Johnson et al., 2016), and the circumvention of hierarchical substrate utilization by abolishing the global regulator protein CRC (Johnson et al., 2017). More recently, a seminal study installed a synthetic genetic module that mediated enhanced catechol consumption and tolerance in *P. putida* KT2440, the presumably most relevant, but must toxic substance among the aromatics (Kohlstedt et al., 2018). An improvement of the set-up for the fed-batch process, which included a regeneration phase without addition of catechol, allowed to achieve a titer of 64 g L^{-1} *cis, cis*-muconate with a basic producer strain MA-1, demonstrating industrial applicability. Additionally, *P. putida* KT2440 MA-9 produced 13 g L^{-1} *cis, cis*-muconate from softwood lignin hydrolysate within 54 h at high yield close to 100% mol mol^{-1} (Kohlstedt et al., 2018) (Fig. 5A).

Besides *P. putida* KT2440, also other microorganisms were shown to be more than suitable for *cis, cis*-muconate production (Table 2). For instance, metabolically engineered *Amycolatopsis* sp. strain ATCC 39116 produced 3.1 g L^{-1} *cis, cis*-muconate from guaiacol within 24 h, and, additionally, produced from a guaiacol-rich lignin hydrolysate (Barton et al., 2018). Less tried and tested in actual applications, the newcomer *C. glutamicum* (Becker and Wittmann, 2019), was stepwise engineered by deleting *catB*, encoding muconate cylcoisomerase, and over-expressing *catA* by the strong, constitutive *eftu*-promoter. The created strain MA-2 reached the world's leading titer of 85 g L^{-1} *cis, cis*-muconate from catechol in 60 h (Becker et al., 2018) (Table 2). In most studies, *cis, cis*-muconate production by biotransformation, relies on glucose as a growth substrate. Despite the renewable character of sugar, this feedstock competes with nutritional purposes (Buschke et al., 2013). Some works, however, imply, that *cis, cis*-muconate-production *via* aromatic biotransformation does not have necessarily to rely on sugars. Next to acetate (Johnson et al., 2017), an interesting approach could be solely based on different aromatics (Sonoki et al., 2018). Here, *P. putida* KT2440 and *Sphingobium* sp. strain SYK-6 were tailored for growth and production from aromatics from softwood (G, H-type-units) or hardwood lignin (S, G, H-type units), respectively (Sonoki et al., 2018). Going on next level, after a detailed screening for bacteria capable to grow on syringate, the chosen *Pseudomonas* sp. strain NGC7 was engineered for *cis, cis*-muconate production from hardwood-lignin (birch) derived aromatics (Shinoda et al., 2019).

7.1.2. Adipate

A major driver for the production of microbial *cis, cis*-muconate production, is that it can be hydrogenated into adipate (Draths and Frost, 1994; Kohlstedt et al., 2018; Vardon et al., 2015, 2016). The enormous relevance of adipate is largely rooted in its function as a building block for the production of nylon-6,6 polyamide (Polen et al.,

2013), corresponding to a global market of $ 6 billion (Joo et al., 2017). In this regard, chemical hydrogenation of *cis, cis*-muconate, bacterially produced from lignin or aromatics, into adipate, subsequent polycondensation with hexamethylene diamine for nylon-6,6-production was successfully shown (Kohlstedt et al., 2018; Vardon et al., 2016), whereby the full value chain starting at softwood lignin was impressively demonstrated by (Kohlstedt et al., 2018) (Fig. 5A). In that course, two independent limited life cycle assessments led to the inference, that bio-based adipate production from lignins can be advantageous over classical petrochemical processes from environmental point of view (Corona et al., 2018; van Duuren et al., 2020), whereby the cost-effectiveness at industrial scale heavily relies on synergistic strain engineering approaches enabling high *cis, cis*-muconate production (van Duuren et al., 2020).

Recent works also prove, that adipate can directly be produced from lignin-based aromatics by exploiting biosynthetic routes (Niu et al., 2020; Suitor et al., 2020). The comprehensive pathway for adipate production using *P. putida* KT2440 started from β-ketoadipyl-CoA, remarkably providing the first direct adipate production from aromatics, while reaching a titer of 2.5 g L^{-1} from 4-hydroxybenzoate in a fermenter-based process (Niu et al., 2020). Promising results were also obtained for an engineered *E. coli*-strain, harnessed for adipate synthesis from guaiacol, relying on different heterologous enzymes (Suitor et al., 2020). Here, *cis, cis*-muconate was sagaciously converted into adipate by an enoate reductase BcER from *Bacillus coagulans* (Suitor et al., 2020), initially described for this purpose in a previous study (Joo et al., 2017).

7.1.3. β-Ketoadipate

Another potential plastics precursor, associated with aromatics metabolism, is β-ketoadipate (Johnson et al., 2019; Okamura-Abe et al., 2016; Suzuki et al., 2021) (Fig. 4). This intermediate not only possesses two dicarboxylic groups, but also a carbonyl group at the beta position (Okamura-Abe et al., 2016) that could be replaced by chemical modification, giving space for modular production of novel nylons with altered properties (Suzuki et al., 2021). β-Ketoadipate production from protocatechuate was shown with a *P. putida* KT2440 mutant harboring an inactivated β-ketoadipate:succinyl-CoA transferase PcaJ (Okamura-Abe et al., 2016). For strain optimization, the mutant was equipped with a plasmid encoding for several key enzymes (*vanAB* (modified *vanA*), *pcaHG, pcaBCD*) under control of the *lac* promoter to ensure inducer-less high-level expression (*P. putida* KT2440-dJ/pKAD) (Okamura-Abe et al., 2016; Suzuki et al., 2021). After process optimization, strain *P. putida* KT2440-dJ/pKAD, with further upgraded expression of vanillin dehydrogenase LigV from *Sphingobium* sp. strain SYK-6, converted each 25 g vanillin and 25 g vanillate at more than 93% yield into β-ketoadipate (Suzuki et al., 2021). Moreover, this strain even converted black liquor obtained from softwood (Japanese cedar) into the target product (Suzuki et al., 2021).

7.1.4. Pyrone- and pyridine-related dicarboxylic acids

2-Pyrone 4,6-dicarboxylate is naturally obtained from the protocatechuate-4,5-*meta* cleavage pathway **(Fig. 4)**, and chemically characterized by a pyran ring and two carboxylic groups (Otsuka et al., 2006). After modification of the carboxyl groups, 2-pyrone 4,6-dicarboxylate could function as a versatile biopolymer precursor (Otsuka et al., 2006), and its potential for polyester production (Michinobu et al., 2008, 2009a, 2009b) has also been demonstrated. In early studies, expression of the *ligAB* and *ligC*-genes from *Sphingobium* sp. strain SYK-6 in *P. putida* PpY1100 provided more than 10 g L^{-1} 2-pyrone 4,6-dicarboxylate from protocatechuate (Otsuka et al., 2006). Follow-up studies showed the suitability of genetically-engineered *P. putida* strains for 2-pyrone 4,6-dicarboxylate production from aromatics derived from kraft, birch and cedar lignin extracts (Qian et al., 2016), and from desulphonated and depolymerized lignosulfonate extracts (Suzuki et al., 2020). Quite recently, *P. putida* KT2440 was shown to simultaneously convert S-, G- and H-type monomers after comprehensive genetic engineering, consisting of a deletion of *pcaHG,* integration of heterologous *ligABC*-genes from *Sphingobium* sp. strain SYK-6, and a replacement of the native *vanAB*-genes by a variant from *Pseudomonas* sp. strain HR199 (Notonier et al., 2021). Apart from *Pseudomonas, N. aromaticivorans* featured by deletions of the *ligI* and *desCD* genes, converted chemically depolymerized poplar lignin into 2-pyrone 4,6-dicarboxylate and, also, reached a high titer (4.9 g L^{-1}) in a fed-batch process, when using vanillate and vanillin as a substrate (Perez et al., 2019).

Elegantly, the protocatechuate-*meta* cleavage pathways were used for the generation of picolinic acid products directly from vanillate, wheat straw lignocellulose and, impressively, kraft lignin (Mycroft et al., 2015). Basically, episomal expression of heterologous *praA*-encoded protocatechuate-2,3-dioxygenase and *ligAB*-encoded protocatechuate-4,5-dioxygenase allowed a rerouting of protocatechuate from the native *ortho*-pathway in *R. jostii* RHA1 (Mycroft et al., 2015). Ammonia cyclization of the extradiol cleavage products generated pyridine-2,5-dicarboxylate (*praA*) or pyridine-2,4-dicarboxylate (*ligAB*), respectively (Mycroft et al., 2015). Building on that, further extensive strain improvement involved (i) the disruption of the competing protocatechuate-3,4-*ortho* cleavage pathway at the level of *pcaHG,* (ii) the integration and constitutive expression of *ligAB*, and (iii) the expression of the lignin-oxidizing enzyme Dyp2 from *Amycolatopsis* sp. for enhanced lignin depolymerization (Spence et al., 2021). By combination of these strategies, a considerable improvement in strain performance was achieved, resulting in 330 mg L^{-1} pyridine-2,4-dicarboxylate from wheat straw lignocellulose (Spence et al., 2021) (Fig. 5C). What's more, for several pyridine-derivatives the actual potential for a completely biobased production of polyesters has already been investigated (Pellis et al., 2019), pointing out also pyridine-2,5-dicarboxylate and pyridine-2,4-dicarboxylate as promising bricks in the precursor portfolio of lignin-derived biomaterials.

7.1.5. Styrene-derivatives

The industrial application potential of styrene-derivatives, e.g., 4-vinylguaiacol, is quite diverse, for instance as flavoring agents in food and perfume industry (Mishra et al., 2014), but also as a potential precursor for biopolymer production (Williamson et al., 2020). With that in mind, microbial production of styrene-derivatives is tempting, whereby several works have focused on the application of *E. coli* as production host (Table 2, Table S1, Supplementary File 2). For example, 237 g L^{-1} 4-vinylguaiacol were obtained from ferulate with heterologous expression of a phenolic decarboxylase from *Bacillus atrophaeus* (L. Li et al., 2019a), whereas in another work the production of 32 g L^{-1} 4-vinylphenol was achieved with a phenolic decarboxylase from *Bacillus amyloliquefaciens* (Jung et al., 2013).

Interestingly, genetically-engineered *P. putida* KT2440 was shown to directly produce 4-vinylguaiacol from industrial soda lignin (Williamson et al., 2020). Chromosomal integration of *padC,* encoding for *B. subtilis* phenolic acid decarboxylase, into the gene locus of *ech* encoding for enoyl-CoA hydratase lyase, not only led to a blocking of native assimilation of phenylpropanoids, but, nicely, allowed a substrate-dependent auto-induced expression of PadC (Williamson et al., 2020). Additionally, the possibility to polymerize 4-vinylguaiacol and 4-vinylcatechol into substituted polystyrenes, generated from ferulate and caffeate using PadC, respectively, was investigated on small-scale with laccase from white-rot fungus *T. versicolor* (Williamson et al., 2020).

7.2. Production of the flavor compound vanillin

Vanillin is, after saffron, the most relevant flavor compound (Banerjee and Chattopadhyay, 2019), and finds copious applications in foods, beverages, cosmetics, perfumes and pharmaceuticals (Banerjee and Chattopadhyay, 2019; Priefert et al., 2001). The limited availability of vanilla pods and consequently, high prices, were important drivers for alternative production routes, relying both on chemical synthesis from guaiacol and biotransformation (Banerjee and Chattopadhyay, 2019;

Priefert et al., 2001). However, regarding environmental issues with chemical synthesis routes, biotechnological production routes have gained in relevance (Banerjee and Chattopadhyay, 2019), also, as they are more conform with customer-driven demands for natural products (Priefert et al., 2001).

Especially, *Amycolatopsis* sp. is considered to be one of the most promising vanillin producers (Fleige et al., 2013; Ma and Daugulis, 2014; Meyer et al., 2017). Building on a profound characterization of vanillin dehydrogenase (Fleige et al., 2013), *Amycolatopsis* sp. strain ATCC 39116 was genetically engineered to enhance its natural-given suitability for vanillin biosynthesis (Fleige et al., 2016) (Fig. 5D). In a first step, natural vanillin degradation was circumvented by deleting the *vdh*-gene encoding for vanillin dehydrogenase, leading to a decrease in vanillin catabolism by nearly 90% (Fleige et al., 2016). The *vdh* deletion mutant F33 was cultivated in a two-step fermentation process and produced 14 g L^{-1} of vanillin after 20 h of biotransformation (molar yield of 81%) (Fleige et al., 2016). Additional improvement targeted at the integration of additional copies of the *fcs* and *ech*-genes, involved in ferulate metabolism, under control of the constitutive promoter *permE** into the former *vdh*-locus (Fleige et al., 2016). The fermentation of strain F84 resulted in 19 g L^{-1} vanillin and a total molar yield of 95%, close to theoretical maximum (Fleige et al., 2016).

Beyond *Amycolatopsis* sp., also other bacteria were shown to be suitable for vanillin production (Table 2, Table S1, Supplementary File 2). Interestingly, *R. jostii* RHA1 was harnessed for vanillin production from wheat straw lignocellulose and kraft lignin (Sainsbury et al., 2013). A *vdh* deletion mutant was thus able to accumulate vanillin in minimal medium, providing 2.5% wheat straw lignocellulose and 0.05% glucose with a titer of up to 96 mg L^{-1} after 144 h (Sainsbury et al., 2013). This study displays an important step towards all-in-one microbial lignin strategies for both lignin depolymerization, and valorization by the same microorganism.

7.3. Polyhydroxyalkanoates

Considering their structural diversity, biodegradability and biocompatibility, polyhydroxyalkanoates (PHA) continuously attract scientific and industrial attention (Prieto et al., 2016; Rehm, 2007; Suriyamongkol et al., 2007). Naturally produced from acetyl-CoA as storage compounds (Rehm, 2007), polyhydroxyalkanoates display linear polyesters, consisting of (*R*)-3-hydroxyalkanoic acids (Prieto et al., 2016; Rehm, 2007; Suriyamongkol et al., 2007). Pseudomonads are well known for the production of PHA (Prieto et al., 2016; Rehm, 2007), and also lignin-derived aromatics, which are metabolized *via* acetyl-CoA (Fig. 4), are suitable for PHA production (Linger et al., 2014). Further metabolic engineering fortified PHA production in *P. putida* KT2440 from aromatics, making use of beneficial genetic targets around the PHA metabolic pathways previously derived from producers that were tailored for other substrates, such as glycerol (Salvachúa et al., 2020a). The undesirable PHA degradation was eliminated by deleting *phaZ* encoding for PHA depolymerase. Sufficient supply of the PHA precursor (*R*)-3-hydroxyacyl-CoA should be guaranteed by (i) deleting *fadBA1* and *fadBAE2* involved in competitory fatty acid β-oxidation, and (ii) overexpressing *phaG* and *alkK* of (*R*)-3-hydroxyacyl-CoA-biosynthesis. Acting in concert with enhancing metabolic flux towards PHA production, the two PHA polymerase-encoding genes *phaC1* and *phaC2* were additionally overexpressed. Applying both *p*-coumarate and, relevantly, a lignin-rich stream derived from corn-stover as a substrate, showed that the resulting mutant strain AG2162 was featured by considerably improved PHA production (Salvachúa et al., 2020a).

A complementary study focused on the metabolic engineering of the aromatic metabolism for improved PHA production, elegantly making the most out of the two catechol cleavage pathways in *P. putida* H (Borrero-de Acuña et al., 2020). In so far, the catechol-*meta* cleavage pathway was assumed to be favorable for PHA production, as it yields stoichiometrically higher levels of the relevant precursor acetyl-CoA than the *ortho*-cleavage pathway. A profound characterization of different *ortho*-cleavage mutants, namely *P. putida* H-Δ*catA*, *P. putida* H-Δ*catA2,* and *P. putida* H-Δ*catAA2,* showed that an outbalanced employment of both catechol degradation routes was essential for advanced substrate uptake, acetyl-CoA production and consequently, PHA synthesis. Thus, the best producer *P. putida* H-Δ*catA2* achieved a PHA titer of 6.1 g L^{-1} in a benzoate-based fed-batch process, and produced 1.4 g L^{-1} from a Kraft lignin hydrolysate (Borrero-de Acuña et al., 2020).

Beyond *P. putida*, also other bacteria were shown to be suitable for PHA-production, and importantly, some works open up the opportunity for streamlined lignin bioconversion and PHA production within the same host (Table S1, Supplementary File 2), including *Cupriavidus basilensis* B-8 (Shi et al., 2017), *Pandoraea* sp. B-6 (Liu et al., 2019), and the marine bacterium *O. doudoroffi* (Numata and Morisaki, 2015).

7.4. Lipids

In the context of biofuels production, huge scientific efforts are invested in microbes with the capability to accumulate intracellular lipids, especially triacylglycerols (Kosa and Ragauskas, 2011; Liang and Jiang, 2013). Triacylglycerols are produced by some bacteria for energy storage, *inter alia Rhodococcus* sp. (Liang and Jiang, 2013). In that course, metabolic supply of acetyl-CoA from β-ketoadipate pathway as a precursor for fatty acid biosynthesis (Kosa and Ragauskas, 2011, 2012) suggested the suitability of *Rhodococcus* sp. based on the combinatorial capability of aromatics-degradation and lipid production (Kosa and Ragauskas, 2011). Thus, *R. opacus* PD630 reached a lipid accumulation of 20.3% of cell dry weight, when grown on *p*-hydroxybenzoate as sole carbon source (Kosa and Ragauskas, 2012). Likewise, when *R. jostii* RHA1 was cultivated on benzoate, lipid accumulation attributed to 55% of its dry weight, which was even enhanced in a mutant overexpressing the transcriptomically-identified diacylglycerol *O*-acyltransferase-encoding *atf8*-gene (Amara et al., 2016).

7.5. Carotenoids

R. rhodochrous has already been described to produce carotenoids (Takaichi et al., 1990), and after an initial screening, *R. rhodochrous* was, also, identified as the most promising producer to make these commercially attractive chemicals accessible for lignin valorization purposes (Chen and Wan, 2021). Optimizing different cultivation parameters revealed interesting synergies between lipid and carotenoid production and enabled a significantly improved production performance, while reaching a carotenoid titer of approximately 1.75 mg L^{-1} (Chen and Wan, 2021). More improvement can be expected given additional metabolic engineering of the so far native producer.

7.6. TCA-related organic acids, dicarboxylic acids, and amino acids

Organic acids are highly in the biotechnological focus (Becker et al., 2015). A putative advantage of using aromatics for the production of TCA-cycle-related compounds, is of course, that they are directly guided into the central carbon metabolism as pyruvate, acetyl-CoA, and succinate ensuring a rich pool of precursors (Elmore et al., 2021) (Fig. 4). *De novo* synthesis of pyruvate and lactate from lignin-related compounds was shown for engineered strains of *P. putida* KT2440, thereby elegantly making use of the natural variability of different ring cleavage pathways and their peculiarities (Johnson and Beckham, 2015). For the accumulation of pyruvate, the pyruvate dehydrogenase complex *aceEF* was deleted, and the highest pyruvate yield (59% wt/wt) could be achieved by implementing the protocatechuate-4,5-*meta* cleavage pathway of *Sphingobium* sp. strain SYK-6 under control of the *tac* promoter (Johnson and Beckham, 2015). In a next step, the gained knowledge was used to realize aerobic lactate production, whereby production was only achieved by replacing the native *ldhA*-gene by a codon-optimized gene

version of an altered lactate dehydrogenase LDHA variant from *Bos taurus* for increased activity. The final strains, additionally carrying a deletion of *lldD* to prevent metabolism of the desired product, enabled a mixed production of pyruvate and lactate from *p*-coumarate (protocatechuate branch) and benzoate (catechol branch) as sole carbon and energy source, respectively (Johnson and Beckham, 2015).

A comprehensive work, recently strived for the production of itaconate (Elmore et al., 2021) (Fig. 5B), which finds, for example, application in the production of detergents and coatings (Becker et al., 2015). Itaconate production was not straightforward and required a combination of different strategies, such as an elimination of the competing polyhydroxyalkanoate production by deletion of $phaC_1ZC_2$, a reduction of the flux through the TCA cycle by exchanging the start codons of *icd* and *idh* encoding for isocitrate dehydrogenase, a testing of two different itaconate production pathways and, the development of an expression system, which should enable a dynamic induction of gene expression, which is limited to the production phase of nitrogen starvation. The highest molar yield of 0.56 mol mol^{-1} was achieved with strain JE3719, which produced 1.26 g L^{-1} itaconate from *p*-coumarate (Elmore et al., 2021).

The presence of aromatics was also shown to have improving impacts on the biosynthesis of certain amino acids using *C. glutamicum* (Lee et al., 2010), but research efforts have not gone further than simple supplementation studies. For instance, supplementing the growth medium with phenol led to an increase in threonine, and histidine production, naphthalene was reported to have a beneficial impact on aspartate production, and additionally, glutamate production was enhanced in the presence of both aromatics (Lee et al., 2010).

8. Conclusions

No matter how tempting, lignin valorization remains undoubtedly a serious challenge. However, the enormous success over the past years, which was probably not to be expected in this amount, is encouraging.

There are several aspects, to be mentioned here: (i) The major strategies for microbial lignin valorization heavily rely on technologies, which are not yet implemented at industrial scale (Johnson et al., 2019). In that context, one crucial factor remains at lignin pre-processing, with the general goal to attain streams which are rich in selected, bioavailable low-molecular weight aromatics (Abdelaziz et al., 2016; Beckham et al., 2016). Nevertheless, the immense scientific efforts invested into microbial lignin valorization, are substantiated by life-cycle assessments (Corona et al., 2018; van Duuren et al., 2020), suggesting environmental benefits over classical petrochemical production routes. Also, in more general terms, technoeconomic analyses go in line with the current aspiration for lignin valorization to ensure economic competitiveness of biorefineries on the long run (Bbosa et al., 2018; Liao et al., 2020). (ii) Microbial lignin valorization profits from the treasure chamber of diverse microorganisms, and different screening methods are ready at hand to make this natural variability accessible for value-added applications (Gonçalves et al., 2020). (iii) The full comprehension of natural lignin degradation and assimilation is the key towards next-level cell factories. Here, also a better understanding of microbial communities in different environments (Díaz-García et al., 2020), (i.e., the group identity) promises valuable insights, in how to make the most out of the available pathways and to specifically tailor microbes in the best-possible manner. (iv) The demanding dual character of aromatics as both substrate and stressor (Mohamed et al., 2020), requires complexly designed bacterial metabolic pathways to tackle these compounds. Meeting the key factors of high yield, productivity and titer, is, thus, closely linked to a facetted employment of different available systems biology tools (Chae et al., 2017). (v) Today, the concept of bacterial lignin valorization is still a “field of dreams” (Becker and Wittmann, 2019). However, it profits vividly from current research, novel discoveries, and innovations, all contributing to turn these ideas at some point into reality and to make the dreams come true.

Declaration of competing interest

Michael Kohlstedt and Christoph Wittmann have filed patent applications on the use of lignin for bio-production. Fabia Weiland declares that she has no competing interest.

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Appendix A. Supplementary data

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