Chapter 6
Biorefining of Lignin Wastes: Modularized Production of Value-Added Compounds

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Abstract Lignin, an aromatic polymer present in lignocellulosic biomasses, is conventionally viewed as a waste by-product of the pulp, paper, and other industries that use plant biomass as feedstocks. More recently, lignin has been reported as a renewable feedstock whose valorization generates several renewable aromatic intermediates, which can be used as carbon sources to synthesize other value-added

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chemicals, polymers, fuels, and other oxidized products. In this endeavor, the lignin's recalcitrance, complex structure, and heterogeneity are major impediments that not only make microbial depolymerization exceptionally challenging but also generate phenolic compounds which inhibit microbial fermentation. Furthermore, during lignin's breakdown, a heterogeneous complex mixture of low-molecular-weight aromatic monomers is released, representing additional hurdles when striving to recover the desired compound selectively. Nevertheless, in nature, some microorganisms are competent to funnel the heterogeneous stream of central aromatic intermediates into a single, pure compound. Centered on lignin, this chapter starts with a generalized description of the structure and types of commercially available lignin (e.g., lignosulphonates or sulfonated lignin, kraft lignin, soda lignin, organosolv lignin, and biorefinery lignin). Next, the slate of aromatic intermediatory compounds formed down the lignin-degrading β-ketoadipate pathway is briefly presented. Finally, this chapter summarizes few case studies related to the production of a high value-added chemical, vanillin, and a biopolymer, polyhydroxyalkanoates (PHAs), using lignin or its derivatives.

Keywords Biorefining · Lignin · Polyhydroxyalkanoates · Valorization · Vanillin

6.1 Introduction

Lignocellulosic biomass (LCB) from agricultural and forest wastes is an abundant renewable feedstock, well-suited for biofuel, biopolymers, and other biomaterials production. LCB is largely composed of cellulose, hemicellulose, and lignin. Normally, cellulose and hemicellulose cover approximately two-thirds of LCB. The remaining material is lignin and its derivatives. Chemical or physiochemical pre-treatment makes the cellulose and hemicellulose fraction readily available for enzymatic saccharification and subsequent production of liquid and gaseous biofuels (e.g., alcohols, hydrogen). Lignin and its derivatives are released as by-products (referred to as "crude lignin"). Annually, the total amount of lignin generated commercially as a bioproduct in biorefineries and other industries such as paper, pulp, and the ethanol industry is estimated to be 10^8 tons (Bajwa et al. 2019; Xu et al. 2019). Much of this lignin is consumed on-site, being formed into pellets for steam and electricity generation. However, when burned as a fuel, crude lignin does not reach its full commercial potential and releases greenhouse gases into the environment. Hence, alternative usage of lignin and its derivatives to synthesize biobased products is desirable and is associated with the lowest ecological impacts. Lignin's valorization for new product development such as aromatic macromolecules, biopolymers, biofuels, and bio-oils is essential to the aging pulp and paper industries, which must expand their commodities portfolio to retain their vitality.

Today, the isolation of lignin from the lignocellulosic biomasses is no longer a barrier, as specific industrial pulping processes—mainly the sulfite, soda, kraft, and organosolv processes—are well established as a technology (see Sect. 6.2 for further

details). However, further depolymerization and fragmentation of lignin into its constituent aromatic building blocks or its derivatives are still a challenge. Lignin is highly heterogeneous and non-uniform in its composition, and its thermochemical valorization leads to the synthesis of multiple product species, necessitating rigorous separation and purification measures to obtain a single target product (Xu et al. 2019). For controlled degradation of lignin, microbial-based methods are becoming increasingly attractive because microbes possess (a) the ability to make and break bonds selectively and (b) diverse metabolic pathways, which can be used to channel lignin's assorted aromatic building blocks, derived intermediates, and other fermentation residuals into a particular target compound. Nevertheless, lignin's depolymerization and subsequent fragmentation release certain phenolics into the media that often inhibit the fermentation and product formation. Furthermore, many of the targeted chemicals are toxic to microbial metabolism. Hence, the use of cell-free systems, such as enzyme extracts or microbial strains having tolerance to the phenolics or aromatic compounds, is being investigated by scientists for attaining the desired conversion rate and the associated molar yields. Efforts to discover alternative microbial pathways that can lead to easy bioconversions while avoiding the generation of side products are also an area of active research.

Today, the production and separation of high added-value compounds from lignin due to their chemistry and properties is an evolving domain of valuable research to both scientific and industrial communities. For sustainable industrial biorefineries, it is important that all the chief components of the lignocellulose, including lignin, be transformed to higher value compounds. This paradigm shift is essential for biorefineries and forestry-based industries to stay competitive. In this chapter, a comprehensive summary of the biological solutions to unlock the potential of lignin for the production of a wide range of bio compounds is presented.

6.2 Structure and Types of Lignin

Lignin is a high-molecular-weight macromolecular found in the cell wall of woods and plants. It is the third largest naturally occurring polymer on the earth after cellulose and chitin (Abdelaziz et al. 2019). Lignin is extremely branched in its structure, composed of three primary phenylpropanoid alcohols (monolignol phenolic units): coniferyl alcohol (G), sinapyl alcohol (S), and p-coumaryl alcohol (H), that are modified with a variety of functional groups such as methoxy ($O–CH_3$), carboxyl (R-COOH), hydroxyl (R-OH) and carbonyl (C=O) (Chatterjee and Saito 2015). There are some complex linkages between the three phenylpropanoid units that provide lignin a dense, hydrophobic structure resistant to depolymerization by enzymes (Abdelaziz et al. 2016; Chatterjee and Saito 2015; Horwath 2015). These include the carbon-carbon bond (C-C), ether bond (C-O-C), C-O bonds of α- and β-arylalkyl ethers, and bonds that OH groups can make with other polysaccharides (cellulose and hemicellulose) and proteins (extensins) in the plant's cell wall. The β-O-4 linkage dominates, represent approximately 45–50% of the linkages, followed

by 5–5 (18–25%), β-5 (9–12%), and the β-1 (7–10%) linkage (Strassberger et al. 2014). While these tight linkages necessitate harsh pretreatments to depolymerize lignin, they make lignin one the most sturdy macromolecule on the planet and are estimated to hold about 95 billion tons of carbon (Chatterjee and Saito 2015). Due to its high carbon content, lignin is considered a significant replacement for fossil fuels. It has also been explored as a renewable bioresource for the synthesis of carbon-based chemicals, and has promising potential as a constituent in polymer blends and composites. However, the ultimate end usage of lignin depends on its properties, which vary according to source and type. Commercially available lignin includes lignosulphonates, kraft lignin, soda lignin, organosolv lignin, and biorefinery lignin.

6.2.1 Lignosulphates

In the industries which separate lignin from cellulose, different oxidative pulping methods are employed. The first and the most widely adopted separation methodology is sulfite pulping, where sulfur dioxide or an acidic bisulfite/sulfite solution is used to soften the plant material and remove lignin as lignosulphonates (Hintz 2001). Lignosulphates are sulfur-containing lignin that behaves as a polar ionic molecule, soluble in water but insoluble in organic solvents. Their single most extensive usage is in the concrete industry, where lignosulphonates are used as plasticizers and allow concrete to be made with 15% less water, which also creates more rigid concrete while retaining its capacity to flow (Gargulak et al. 2015; Vazquez and Pique 2016). Lignosulphonates have the power to decrease the viscosity of the solutions and provide hydration. They also find usage in the production of linoleum flooring and plaster (Vazquez and Pique 2016). Lignosulphonates possess good binding, dispersing, and emulsifying abilities that make them useful additives in tanning, leather, pesticides, fertilizers, *wax emulsions, dyes, pigments, oil drilling mud,* coal briquettes, *and food industry (*WebArchive 2003*).*

Chemical oxidation of sulfonated lignin at elevated temperature (up to 160 °C) and pressure (10 bar) has a value for the production of low-molecular-weight artificial flavoring agents such as vanillin and vanillic acid (4-hydroxy-3-methoxy benzoic *acid*) (Pacek et al. 2013; Richter et al. 1945). The global market potential of vanillic acid (in 2020) was USD 1189.9 million (Fox40 2020). Dimethyl sulphoxide (DMSO) is another high-utilization chemical derived from lignosulphonates (Macfarlane et al. 2014). Hence, lignosulphonates have immense market potential. However, the sulfite coking process by which they are produced is environmentally costly, and lignosulphonates contain sulfur and hemicellulose (Abdelaziz et al. 2016), making them less pure. It is their property of being soluble in water that distinguishes them from other lignin streams. Due to their anionic sulfate groups, lignosulphonates can scavenge metals and keep them dissolved in solutions. This property has an advantage in agriculture where lignosulphonates can keep metals available to plants (preventing them from precipitating out as insoluble compounds), and also in drinking water systems, where scaly metals deposition on the walls of the

water systems can be avoided (WebArchive 2003). Moreover, with their ability to immobilize metals, the utilization of lignosulphonates for bioremediation of wastewater cannot be ruled out.

6.2.2 Kraft Lignin

Compared to sulfite pulping, the more modern pulping process is the Kraft process, where the plant material is treated with a mixture of water, sodium hydroxide (NaOH), and sodium sulfide (Na_2S) at 140–180 °C to break the linkages binding lignin with cellulose and hemicellulose. The lignin extracted during this process is known as Kraft lignin which accounts for about 85% of the total lignin production in the world (Chen 2015). Approximately 630,000 tons of kraft lignin is produced annually, and most of its utilization is for combustion. Its high value utilization as an additive and binder for improvement of the properties of resins, foams, printing inks, fertilizers, and adhesives, only stands at 2% of the total kraft lignin produced (Macfarlane et al. 2014). Like lignosulphonate, kraft lignin has reactive sulfate groups attached to its phenyl rings. Also, it is associated with hemicellulose, making it impure. Nevertheless, there is an ongoing push to use kraft lignin for producing activated carbon and as a low-cost raw material for carbon fiber synthesis. It has an expected compound annual growth rate (CAGR) of 7% (John 2020).

6.2.3 Soda or Alkaline Lignin

Produced by one of the simplest pulping processes, soda or alkaline lignin is produced as a by-product in the pulp industry using sodium hydroxide (NaOH) or a mixture of NaOH and anthraquinone at 150–170 °C (Macfarlane et al. 2014). On average, soda pulping yields approximately 80% of lignin from the wood samples (USDA 2020). Performing a steam explosion before soda pulping can enhance the lignin extraction percentage to 90% (USDA 2020). While the lignin from this process is sulfur-free, it still contains hemicellulose. It has comparatively high aliphatic and phenolic content than lignosulphonates and kraft lignin (Abdelaziz et al. 2016). Hence, aromatic resins are the key chemicals expected to be derived from the use of soda lignin as the substrate. Also, because they are free from sulfur, soda lignin can be utilized as binders in animal feed (Macfarlane et al. 2014).

6.2.4 Organosolv Lignin

Organosolv is the most eco-friendly pulping process that uses organic solvents (e.g., ethanol, acetone, methanol, acetylene glycol, etc.) to efficiently depolymerize wood

into lignin, hemicellulose, and cellulose fractions. Organosolv lignin is of high quality and purity, with <1 wt.% residual carbohydrate content (Strassberger et al. 2014). Organosolv lignin is free from sulfur and other impurities. Hence, it can directly be used to produce specialty value-added products via a more environmentally friendly method. Organosolv lignin has a highly homogenous nature, with its composition being much closer to native lignin (Tribot et al. 2019). It is very rich in phenolic content and is highly hydrophobic, which allows it to be spun into fibers directly, without blending with other polymers (Macfarlane et al. 2014). Also, because of its purity, organosolv lignin can be exploited for its antioxidant, antibiotic, and antitumor properties in the cosmetics, medicinal, and pharmaceutical sectors (Macfarlane et al. 2014). To date, organosolv lignin is the most attractive lignin in terms of its quality. However, the commercial realization of the organosolv processes is presently marginal, probably as a result of high process costs, and is not readily available at volume (Abdelaziz et al. 2016; Thoresen et al. 2020).

6.2.5 Biorefinery Lignin

The lignin produced as a by-product in biorefineries using second-generation lignocellulosic feedstocks is another source of lignin, with an annual production of approximately 100 kilotons (Bajwa et al. 2019). The biorefinery lignin, produced by a hydrolytic pretreatment (acidic, thermal, or enzymatic) of underutilized lignocellulose is essentially sulfur-free. However, some hemicellulose is still linked with it through ester, glycosidic, ether, or carbon-carbon bonds (Hansen et al. 2013). These bonds are responsible for the hydrophilic surface properties of lignin, which limits its applications in some industries, including the range of polymer resins it can be effectively combined within biocomposite applications. Nevertheless, biorefinery lignin holds the potential to be precipitated from the rest of the biomass in the form of an insoluble, amorphous, solid residue that has a significant amount of protein attached to it and that can be used as animal feed (Hansen et al. 2013). In the future, removing carbohydrate impurities and polishing lignin by enzymes could yield low-molecular-weight lignin with higher purity and hydrophobicity suitable for various industrial applications.

Over the years, lignin separated from lignocellulosic biomass by different biomass conversion technologies has been given distinct names, as in cellulolytic enzyme lignin, produced by cellulolytic enzyme treatment of pretreated agricultural residue (Tian et al. 2017; Zhang et al. 2010); Bjorkman lignin, produced by treatment of lignocellulose with neutral organic solvents (Bjorkman 1954; Obst and Kirk 1988); Klason lignin, produced by treatment of lignocellulose with sulphuric acid followed by removal of ash (Chen 2015; Obst and Kirk 1988), etc. (Retsina et al. 2013).

From this section, it is evident that varieties of industrial lignins exist in the market whose properties and structures depend on the pulping or the coking process (Table 6.1). These lignins are directly suitable for a range of applications, from

Table 6.1 Comparison of lignins generated from lignocellulosic via various cooking processes

Industrial lignin	Molecular Weight (kDa) (USDA 2020)	Impurity	Sulfur content (%) (Abdelaziz et al. 2016)	Hemicellulose content	Phenolic content	Solubility in organic solvents	Worldwide Production (kilotons/year) (Miller 2016)	Price (USD/ton) (2019) (Tribot et al. 2019)
Lignosulfonates	20,000–50,000	Sulphur, and hemicellulose	5–6	High	Low	Insoluble. Soluble in water	1000	300–2700
Kraft lignin	100–3000	Sulphur, and hemicellulose	1–2	High	Low	Insoluble. Soluble in water above pH 11	75	250–500
Soda or alkaline lignin	800–3000	Hemicellulose, ash	Nil	Average	High	Partly soluble	5–10	200–300
Organosolv lignin	500–4000	None	Nil	Very low (<1%)	Highest	Highly soluble	3	280–520
Cellulolytic lignin	Variable	Hemicellulose, ash	Nil	High	Variable	Insoluble	100	Not determined

low-density combustion fuel to binder and blender additives to make high-strength and durable concrete, cardboard, and papers. Lately, the use of lignin in its purest form as an antioxidant has been recognized. Furthermore, in 2007, scientists from the Pacific Northwest National Laboratory (PNNL) released a report evaluating the opportunity for using lignin for the derivation of certain macromolecules, higher aromatic monomers, and oxidized compounds by breaking the lignin's polymeric structure (Holladay et al. 2007). The report presented a case that lignin produced in biorefineries has a high economic opportunity to generate large revenues (USD 12–35 billion) by producing a variety of co-products (Holladay et al. 2007). This strategy would entail the choice of technology for selective fragmentation of lignin into low molecular weight monomers and oligomers, which can be further biocatalyzed by microorganisms, via cellular assimilation, into value-added compounds.

Frequently, the microorganisms that depolymerize lignin into its constituent monolignols proceed to polymerize these intermediates into other renewable chemicals using their versatile metabolic pathways. Section 6.3 touches on some of the high-value lignin monomers and oligomers synthesized by microbial/enzymatic-based lignin depolymerization without going into the technical details of these processes. Section 6.4 discusses some final value-added chemicals produced by microorganisms via cellular assimilation of such lignin intermediates via the β-ketoadipate pathway.

6.3 Mono-and Oligomers as Intermediates from Lignin Depolymerization

In nature, microorganisms existing in symbiotic association with plants have evolved mechanisms to degrade and utilize lignocellulosic biomass. These plant degrading microbes release a repertoire of extracellular enzymes collectively termed ligninolytic enzymes (comprising laccase, superoxide dismutase, oxidoreductases, and peroxidases) that oxidize phenolic units in lignin, but not the non-phenolic compounds (Datta et al. 2017; Govil et al. 2020a; Janusz et al. 2017). Some mediators such as p-coumaric acid, 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid, vanillin, and syringaldehyde, can enhance the oxidative capacity of laccase itself to oxidize the non-phenolic units in lignin (Abdelaziz et al. 2016). In addition, some low-molecular-weight secondary metabolites produced by microbes during lignin degradation, such as benzoic acid, veratryl alcohol (MnO_2), oxalate, and 2-chloro-1,4-dimethoxybenzene, can aid the further breakdown of the phenolic and non-phenolic groups in lignin (Datta et al. 2017; Janusz et al. 2017; Shimada et al. 1981). These metabolites are slowly metabolized by the microorganisms and accumulate in the reaction solutions. Other metabolites such as flavonoids, tannins, and lignans are parts of plants themselves. They are also known to initiate bond

scissions in lignin via an oxidative and reductive cascade of reactions (Janusz et al. 2017).

Over the years, many studies have reported the biodegradation of lignin by microorganisms and their enzymes, and significant progress has been made in understanding these processes (Abdelaziz et al. 2016; Lee et al. 2019). Literature is also available that details the pathways for lignin valorization. The pathways suggest that during the breakdown of lignin, various low-molecular-weight aromatic compounds can be formed depending on the composition of the lignin and the depolymerization method used. The most common of these monolignols are p-coumaric acid, caffeic acid, ferulic acid, guaiacol, syringic acid, syringaldehyde, phenol, benzoic acid, and vanillic acid (Abdelaziz et al. 2016). Tang et al. (2015) reported production of 8.04 mg, 0.88 mg, 0.63 mg, 0.34 mg, and 0.29 mg of hydroxybenzoic acid, syringaldehyde, vanillic acid, *p*-coumaric acid, and ferulic acid, respectively, from each gram of oil palm empty fruit bunch lignin. Chen et al. (1982) reported the generation of vanillic acid, veratric acid, and various benzoic acid derivatives during degradation of spruce wood lignin by white-rot fungus *Phanerochaete chrysosporium* (Chen et al. 1982). The production of phenolic oligomers containing about seven phenylpropane units from kraft lignin when subjected to degradation by the fungus *Trametes versicolor* has also been reported (Reid 1998). Guaiacol, benzoic acid, and vanillic acid were identified as significant intermediates when a member of the genus *Acetoanaerobium* degraded kraft lignin. In the same study, ferulic acid, syringic acid, and benzenepropanoic acid were also detected, which were considered the final products after intermediate stage degradation (Duan et al. 2016). Consistent with these observations, similar degradation products have also been found in other studies, some of which are summarized in Table 6.2.

Commercially, many of these degradation products of lignin are valued chemicals, and they can find industrial applications. For instance, syringaldehyde is an aromatic aldehyde with valued antioxidant, bioactive (antimicrobial), and antioncogenic activity and is used in cosmetics, pharmaceuticals, food, paper, and pulp industries. Moreover, syringaldehyde is also a promising laccase and peroxidase mediator that can enhance these enzymes' activity by almost six-fold (Mohamad Ibrahim et al. 2012). p-coumaric acid possesses excellent anti-infection, anti-inflammatory, and antioxidant activities that can help it protect against conditions of oxidative stress (Shen et al. 2019). Ferulic acid also possesses antithrombic, antimicrobial, anticancer, antidiabetic, and immunostimulant properties, and finds applications in cosmetics, pharmaceuticals, food, and health industries (Kumar and Pruthi 2014). Guaiacol and its derivatives are valuable as additives in mucoactive, antiseptic, and anesthetic agents. Indeed, lignin and the phenolic compounds derived from it have a total market value of approximately USD 732 million, projected to reach USD 913 million by 2025 (Bajwa et al. 2019). However, despite the potential to synthesize such promising aromatics, vanillin stays at present the only marketable aromatic product of lignin produced using microbial sources.

Table 6.2 Aromatic intermediary metabolites produced from biological lignin depolymerization

Source of lignin	Microbe/enzyme	Conditions	Product	Reference
Oil palm empty fruit bunch	Cutinase and manganese peroxidase	55 °C, pH 8.0	Hydroxybenzoic acid, syringaldehyde, vanillin, *p*-coumaric acid, and ferulic acid	Tang et al. (2015)
Spruce wood	*Phanerochaete chrysosporium*	30 °C, pH 7.0	Vanillic acid, veratric acid, and benzoic acid	Chen et al. (1982)
Kraft lignin	*Trametes versicolor*	40 °C, pH 4.8	Seven phenylpropane oligomer	Reid (1998)
Kraft lignin	*Acetoanaerobium* sp. WJDL-Y2	40 °C	Ferulic acid, syringic acid, and benzenepropanoic acid	Duan et al. (2016)
Biorefinery lignin	*Pseudomonas putida* and *Rhodococcus* RHA1	30 °C	Propiophenone and benzoic acid derivatives	Ahmad et al. (2010)
Kraft lignin	*Bacillus* sp. and *Aneurinibacillus aneurinilyticus*	55 °C, pH 7.0	Trans-4- hydroxycinnamic acid, 3,4,5-trimethoxy benzaldehyde, gallic acid and ferulic acid	Raj et al. (2007)
Kraft lignin	*Comamonas* sp. B-9	30 °C, pH 7.0	Ethanediol, 3, 5-dimethylbenzaldehyde and phenethyl alcohol	Chen et al. (2012)
Kraft lignin	*Dysgonomonas* sp. WJDL-Y1	33 °C, pH 6.8	Vanillic acid, syringic acid, ferulic acid, and benzoic acid	Jing et al. (2016)
Wastepaper	*Aeromonas formicans*	30 °C, pH 7.2	Benzoic acid, Vanillic acid, Protocatechuic acid, Syringic acid, cinnamic acid and ferulic acid	Gupta et al. (2001)
Milled wood lignins	β-O-4-cleaving enzymes from *Novosphingobium sp.*	15 °C, pH 8.5	Guaiacylhydroxylpropanone and Syringylhydroxylpropanone	Ohta et al. (2017)
Softwood lignin	*E. coli* recombinantly expressing β-O-4-cleaving enzymes	30 °C, pH 8.0	Vanillin	Reiter et al. (2013)
Wheat straw lignocellulose	Mutant *Rhodococcus jostii* RHA1	30 °C, pH 8.0	Vanillin	Sainsbury et al. (2013)

6.4 Final Value-Added Chemicals Production from Lignin Intermediates

Ideally, the production of these lignin intermediates is linked to each other via the β-ketoadipate pathway. One intermediate gets converted to the other rapidly via a cascade of reactions. Hence, most of the time, lignin breakdown is a complex mixture of low-molecular-weight aromatic monomers. It is, in fact, difficult to

synthesize a particular aromatic compound from lignin at sufficient concentration. This has a negative prospect for the valorization of lignin to generate a definable aromatic compound. However, Ohta et al. (2017) showed that highly specific aromatic monomers can be synthesized from lignin by controlling the reactions using selective enzymes. In their study, the authors achieved the exclusive synthesis of monomers with a phenylpropane moiety (e.g., guaiacylhydroxylpropanone (GHP), syringylhydroxylpropanone, SHP)) using four β-O-4-cleaving enzymes (two short-chain dehydrogenase/reductase, two glutathione *S*-transferases with β-etherase activity) isolated from a *Novosphingobium* strain in one pot (Ohta et al. 2017). Prim et al. (2003) showed that the production of 4-ethyl phenol, a phenolic compound responsible for aroma in wine, is possible from hydroxycinnamic acids (e.g., ferulic, p-coumaric, sinapyl acid, and caffeic acids) using phenolic acid decarboxylase from *Bacillus* sp. BP-7, with no accumulation of any side products (Prim et al. 2003). A similar conversion was achieved using a decarboxylase gene from *Bacillus licheniformis* (Hu et al. 2015). Furthermore, phenolic acid decarboxylase activity from *Bacillus amyloliquefaciens* has been demonstrated for the selective synthesis of p-hydroxystyrene from p-coumaric acid (Jung et al. 2013).

6.4.1 Specific Case of Vanillin Production

As mentioned earlier, because of lignin's heterogeneity, many side products are formed along with the desired compound during lignin processing and intermediate production. To avoid this issue, studies have been conducted to examine microbial enzymes which can bioconvert a particular metabolite into other monomeric aromatic compounds. In this regard, processes that lead to the production of vanillic acid are one of the most intensively studied enzymes and enzymatic processes. Vanillic acid is a food preservative and a natural precursor for vanillin production - an aromatic compound responsible for the characteristic vanilla flavor, which has enormous consumer demand. Organic vanillin obtained from plants is high-priced (approximately USD 3000/kg) and has a net worth of more than USD1 billion annually (Li and Rosazza 2000). Vanillin synthesized chemically is comparatively cheaper (USD 11/kg) though it is not considered natural under US legislation (Ashengroph et al. 2011). Hence, cheaper production of natural vanillin using microbial transformations has increasingly been attempted and has immense economic potential, owing to the spur in demand of bio-vanillin as a flavoring base in foods, pharmaceuticals, and cosmetic industries (Luziatelli et al. 2019) (Fig. 6.1).

For years, the most exploited feedstock for vanillic acid's biological production is ferulic acid (a hydroxycinnamic acid found attached to hemicellulose via several ester linkages). Some of the microbial enzymes that have been studied for this purpose include enzymes belonging to the superfamily feruloyl enoyl-SCoA hydratase/lyase (EC 4.2.1.101) and feruloyl-CoA synthetase (EC 6.2.1.34)) expressed by *Pseudomonas fluorescens* (Gasson et al. 1998; Leonard et al. 2006), *Pseudomonas* sp. Strain HR199 (Overhage et al. 1999), *Pseudomonas putida*

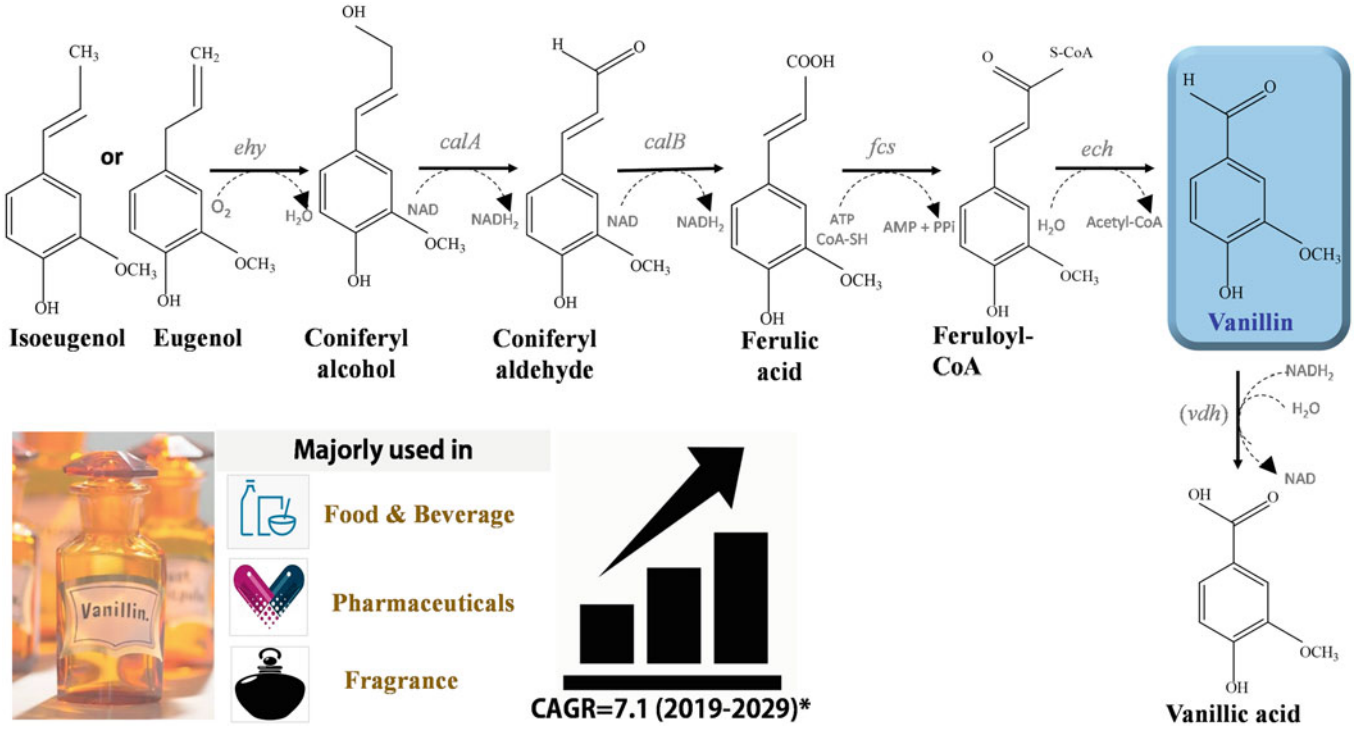

Fig. 6.1 Production of vanillin from Ferulic acid and Isoeugenol. *ehy* Eugenol hydroxylase, *calA* Coniferyl alcohol dehydrogenase, *calB* Coniferyl aldehyde dehydrogenase, *fcs* Feruloyl-CoA synthetase, *ech* Enoyl-SCoA hydratase/lyase, *vdh* Vanillin dehydrogenase. (Source of data depicting compound annual growth rate of Vanillin: Persistence Market Research Report, released Match 2020 (PMR 2020))

KT2440 (Plaggenborg et al. 2003), plant species *Glechoma hederacea* and *Vanilla planifolia* (Gallage et al. 2014; Negishi et al. 2009), *Amycolatopsis* sp. strain HR167 (Achterholt et al. 2000), *Streptomyces* sp. NL15-2K (Nishimura et al. 2018; Yang et al. 2013) and strain V1 (Hua et al. 2007b), *Streptomyces setonii* (Muheim and Lerch 1999), *Delftia acidovorans* (Plaggenborg et al. 2001), *Rhodotorula rubra* (Huang et al. 1993), *Halomonas elongate* (Abdelkafi et al. 2006), *Bacillus subtilis* (Gurujeyalakshmi and Mahadevan 1987), and *Aspergillus niger* CGMCC0774 and *Pycnoporus cinnabarinus* CGMCC1115 (Zheng et al. 2007). In the two-step conversion process, feruloyl-CoA synthetase (*fcs*) converts ferulic acid into feruloyl CoA and subsequently enoyl-SCoA hydratase/lyase (*ech*) mediates the conversion of feruloyl CoA to vanillin and acetyl CoA (Fig. 6.1). Table 6.3 summarizes the studies where the natural host strain or the recombinant strains expressed the enzymes that could transform ferulic acid to vanillin and yields up to 28.3 g/L were achieved. In the future, cloning an efficient ferulic acid esterase gene that can release ferulic acid from hemicellulose (Bugg et al. 2011) in such clones can create a path for producing vanillin directly from hemicellulose.

As such, ferulic acid is an excellent nontoxic precursor for vanillin synthesis. A high concentration of ferulic acid can be fed to the microorganisms without inhibiting microbial growth (Muheim and Lerch 1999). However, ferulic acid is costly, and this has prompted research where eugenol (a plant-derived phenylpropanoid) is being considered as a cheaper (USD 5/kg) and a more abundant vanillin precursor. Several microbial strains and their enzymes have been shown to aid in the biotransformation of eugenol into vanillin, including *Rhodococcus rhodochrous* (Chatterjee et al. 1999), *Serratia marcescens* DSM30126 (Rabenhorst and Hopp 1991), strains of the genus *Bacillus* (Hua et al. 2007a; Shimoni et al. 2000; Zhang et al. 2006), *strains of the genus Pseudomonas* (Kasana et al. 2007; Unno et al. 2007; Yamada et al. 2007), and *Candida galli* strain PGO6 (Ashengroph et al. 2011). Lately, the use of cell-free extracts rich in characteristic enzymes for biocatalysis of eugenol into vanillin has also been attempted. For example, vanillyl alcohol oxidase enzyme (vaoA) from *Penicillium simplicissimum* CBS 170.90 has successfully been used for the biocatalytic conversion of 1 g/L of eugenol into vanillin (0.24 g/L, the molar yield of 10%) and vanillic acid (1.1 g/L, the molar yield of 44%) (Ashengroph et al. 2011). *vaoA* (Vanillyl-alcohol oxidase) gene when cloned into a recombinant *Escherichia coli*, that had already been transformed with the genes encoding coniferyl alcohol dehydrogenase and coniferyl aldehyde dehydrogenase from *Pseudomonas* sp. strain HR199, produced 0.3 g of vanillin per liter of the fermentation medium from eugenol (Overhage et al. 2003).

Several attempts have been made to produce vanillin with a minimum of co-products from lignin as the starting substrate. For example, Reiter et al. (2013) recombinantly expressed three β-O-4-cleaving enzymes, a Cα-dehydrogenase, a β-etherase, and a glutathione lyase from the proteobacterium *Sphingobium* sp. *SYK6* in *E. coli* BL21, and obtained the production of 58.2 mg/L of vanillin from softwood lignin with a small amount of ferulic acid as the co-product (Reiter et al. 2013). Exclusive production of vanillin from wheat straw lignocellulose was also reported in a study by Sainsbury et al. (2013), where a mutant strain of

Table 6.3 Case studies detailing vanillin production by biological means from lignin or its intermediates

Host/Enzyme	Substrate	System	Conditions	Vanillin concentration (g/L)	Substrate concentration (g/L)	Time	Molar yield (%)	References
Aspergillus niger CGMCC0774 and *Pycnoporus cinnabarinus* CGMCC1115	Ferulic acid	Growing cells	30 ° C, pH 7.2, 150 rpm	2.2	4	72	57.7	Zheng et al. (2007)
Streptomyces sp. strain V1	Ferulic acid	Growing cells	Fed-batch 30 ° C, pH 5.8, 200 rpm	19.2	9	18	58	Hua et al. (2007b)
Streptomyces setonii	Ferulic acid	Growing cells	Fed-batch 30 ° C, pH 7.2, 150 rpm	6.41	12	54	68	Muheim and Lerch (1999)
Bacillus subtilis	Ferulic acid	Biofilm	35 °C, pH 9.0, 200 rpm	1.84	2	20	60.43	Yan et al. (2016)
Halomonas elongate	Ferulic acid	Resting cells	37 ° C, pH 7.2, 150 rpm	0.65	0.97	14	86	Abdelkafi et al. (2006)
Recombinant *Escherichia coli* XL1-Blue	Ferulic acid	Resting cells	30 ° C, 150 rpm	0.3	5	15	ND	Overhage et al. (2003)
Recombinant *E. coli* strain JM109	Ferulic acid	Resting cells	30 ° C, 150 rpm	2.52	0.21	6	50	Barghini et al. (2007)
Recombinant *E. coli* DH5alpha	Ferulic acid	Growing cells	37 ° C, 180 rpm	5.14	3.0	24	86.6	Lee et al. (2009)
Recombinant *E. coli* NTG-VR1	Ferulic acid	Growing cells	37 ° C, 180 rpm	2.9	10	48	62%	Yoon et al. (2007)
Recombinant *E. coli* FR13	Ferulic acid	Resting cells	30 ° C, pH 9.0	4.2	4.5	24	68%	Luziatelli et al. (2019)
Engineered Amycolatopsis sp. ATCC 39116	Ferulic acid	Growing cells	Fed-batch 45 ° C, 600 rpm	22.3	10	20	94.9	Fleige et al. (2016)

Recombinant *Pseudomonas putida* KT2440	Ferulic acid	Resting cells	30 ° C	1.30	2.0	3	86%	Graf and Altenbuchner (2014)
Recombinant *P. fluorescens* BF13 3	Ferulic acid	Growing cells	3 L stirred tank reactor 30 ° C, 200 rpm	1.28	2.0	24	81%	Di Gioia et al. (2011)
Recombinant *Pediococcus acidilactici* BD16	Ferulic acid	Growing cells	37 ° C, 150 rpm	0.48	0.2	0.33	ND	Kaur et al. (2014)
Pseudomonas putida IE27	Isoeugenol	Resting cells	20 ° C, 200 rpm with 10% DMSO	16.1	24	24	71	Yamada et al. (2007)
Candida galli strain PGO6	Isoeugenol	Resting cells	30 ° C, pH 7.0, 200 rpm	1.12	5	60	25.7	*Candida galli* strain PGO6
Pseudomonas resinovorans SPR1	Isoeugenol	Resting cells	28 ° C, 200 rpm	0.24	1	30	10	Ashengroph et al. (2011)
Psychrobacter sp. strain CSW4	Isoeugenol	Resting cells	28 ° C, 200 rpm	13.8	10	48	10	Ashengroph et al. (2012)
Pseudomonas chlororaphis CDAE5	Isoeugenol	Growing cells	25 ° C, 180 rpm	1.2	10	24	12.6	Kasana et al. (2007)
Trichosporon asahii	Isoeugenol	Growing cells	28 ° C, pH 5.8, 200 rpm	2.4	5	24	52.5	Ashengroph and Amini (2017)
Soybean lipoxygenase	Isoeugenol	Enzyme extract	28 ° C, pH 9.0, 180 rpm	2.68	30	120	ND	Liu et al. (2020)
Halomonas sp. B15	Isoeugenol	Growing cells	33 ° C, pH 7.0, 150 rpm	0.365	1.5	112	36.5	Vyrides et al. (2015)
Recombinant *E. coli* (BL21)	Isoeugenol	Growing cells	20 ° C, 180 rpm	28.3	37	6	81	Yamada et al. (2008)
Recombinant *E. coli* BL21	Softwood lignin	Growing cells	30 °C, pH 8.0	0.089	25	50	ND	Reiter et al. (2013)

(continued)

Table 6.3 (continued)

Host/Enzyme	Substrate	System	Conditions	Vanillin concentration (g/L)	Substrate concentration (g/L)	Time	Molar yield (%)	References
Engineered Rhodococcus jostii RHA1	Wheat straw lignin	Growing cells	30 ° C, pH 8.0, 180 rpm	0.096	25	144	ND	Sainsbury et al. (2013)

DMSO Dimethyl Sulfoxide, *ND* Not determined

Rhodococcus jostii RHA1 deficient in gene vanillin dehydrogenase was found to accumulate up to 96 mg/L of vanillin, together with minor quantities of ferulic acid and 4-hydroxybenzaldehyde (Sainsbury et al. 2013). These studies show that the exclusive production of specific phenolic compounds from lignin is possible, provided the application of targeted pathway engineering can control the biocatalytic routes for lignin breakdown. However, the reported yield and concentration of products produced via this route are below 1 g/L and do not qualify as economically efficient biotransformation. In these studies, the reported yield of vanillin production of less than 1 g/L and associated efficiency of less than 10%, was due to the toxic effects of vanillin on the cellular systems at concentrations above 1 g/L, leading to its quick metabolization by the microorganism into vanillic acid and vanillyl alcohol using vanillin dehydrogenase (EC 1.2.1.67) (Ashengroph and Amini 2017). Hence, in microbial hosts, the respective alcohol or the respective acid typically gets accumulated rather than vanillin.

To enhance the bioconversion yield of vanillin from eugenol or ferulic acid, the use of static growth conditions is the first strategy adopted where resting cells have been shown to delay degradation of vanillin to vanillic acid. Here, with the resting cells of *Psychrobacter* sp. strain CSW4, a vanillin concentration of 1.28 g/L (molar yield of 13.8%) was achieved by Ashengroph et al. (2012). Secondly, researchers have also, from time to time, reported the isolation of a few strains from nature that are resistant to the toxic effects of vanillin, and hence can accumulate more vanillin in a reduced reaction time. *Pseudomonas chlororaphis* CDAE5 is one such strain that has demonstrated the potential to be a suitable candidate for biotechnological production of vanillin from isoeugenol. Another study has shown *Pseudomonas chlororaphis* CDAE5 to produce 1.2 g/L of vanillin, with a molar yield of 13% (Kasana et al. 2007). Much higher productivity of vanillin has been reported by Ashengroph and Amini (2017), where the yeast *Trichosporon asahii* transformed 5 g/L of isoeugenol into 2.4 g/L of vanillin with a 52.5% molar yield (Ashengroph and Amini 2017). Even cell-free extracts rich in a designated enzyme have been investigated for the process. Enzyme lipoxygenase is useful for this biocatalysis approach, where Liu et al. (2020) tested a method for the synthesis of vanillin from isoeugenol and eugenol using soybean lipoxygenase (lipoxidase). The reported production of vanillin in this study was 2.68 g/L. A European patent dating back to 1991 claimed a process for the preparation of vanillin (10–15 g/L) from eugenol or isoeugenol using lipoxidase (Markus et al. 1992), an enzyme from *Glycine max* (soybean) that is now commercially available from Sigma Aldrich (L6632 and L7395).

To augment these processes and improve productivity, the metabolic engineering of microbial strains already known to have the tolerance to vanillin toxicity has been documented in the literature. Specifically, *Amycolatopsis* sp. ATCC 39116, a gram-positive Actinobacteria, was engineered with the deletion of its vanillin dehydrogenase-encoding (vdh) gene that codes for vanillin catabolism enzyme, vanillin dehydrogenase. This mutation decreased the catabolism of vanillin to vanillic acid by 90%, and resulted in an increase of the total vanillin production to 2.2 g/L from ferulic acid, with a molar yield of 80.9% (Fleige et al. 2016). The same group

achieved a vanillin concentration of 19.3 g/L (molar yield of 94.9%) by constitutively expressing two of the vanillin anabolism genes *fcs* (coding for feruloyl-coenzyme A (CoA) synthetase) and *ech* (enoyl-CoA hydratase/aldolase) in the same stain of *Amycolatopsis* sp. 39116 (Fleige et al. 2016). The transcription of *ech* and *fcs* eliminated the adaptation phase in the host. Moreover, by using an improved fed-batch feeding strategy, the group could attain an even higher concentration of vanillin, 22.3 g/L, which is the highest vanillin concentration reported to date from any of the native wild host strains (Fleige et al. 2016). This study shows that improvements in vanillin yield using whole cells are possible through the right combination of strategies involving optimization of the fermentation parameters with resting cells and metabolic engineering. The identical strategy of inactivating vanillin dehydrogenase and overexpressing feruloyl-coenzyme A (CoA) synthetase and enoyl-CoA hydratase/aldolase in *Pseudomonas* sp. have been documented by Graf and Altenbuchner (2014). However, here the authors observed vanillin metabolism to vanillic acid, despite knockout of the vanillin dehydrogenase gene (*vdh*). Hence, additional inactivation of a molybdate transporter gene was done in their study, which led to the complete prevention of vanillin degradation. However, the concentration of vanillin achieved in their study was only 1.2 g/L (Graf and Altenbuchner 2014). This indicates that each microbial strain has a characteristic tolerance to the amount of vanillin it can produce in the system. The highest concentration of vanillin production of vanillin has been achieved at 1.2 g/L with any *Pseudomonas* strain to date.

Genetic engineering strategies have also been tried with recombinant *E. coli* (transformed with vanillin synthesizing genes) as the preferred candidate for cost-effective vanillin synthesis because it has a well-studied and understood fermentation process and has no vanillin degradation pathway (Lee et al. 2009). Lee et al. induced an *E. coli* host transformed with feruloyl-CoA synthetase (*fcs*) and enoyl-CoA hydratase/aldolase (*ech*) genes to produce more vanillin by amplifying a *glt* gene encoding citrate synthase in it. During the vanillin synthase from ferulic acid, acetyl-CoA is a concomitant by-product whose accumulation impedes feruloyl-CoA's forward reaction to vanillin. The enzyme citrate synthase bio transforms acetyl-CoA into CoA and helps the vanillin synthesis reaction to be pulled forward by eliminating product inhibition. Therefore, in their study, by overexpressing the *gltA* gene, 1.98 g/L of vanillin was produced, which was almost twofold more than the vanillin production of 0.91 g/L obtained by the *E. coli* without *gltA* amplification (Lee et al. 2009). In another study, Yoon et al. (2007) followed a two-step strategy to enhance vanillin production in an *E. coli* harboring *fcs* and *ech* genes transformed from *Amycolatopsis* sp. strain HR104. First, they generated mutants of *E. coli* that were vanillin resistant, and second, they used XAD-2 resin for the adsorption and removal of released toxic vanillin from the medium. This combined engineering strategy increased the vanillin production from the recombinant host to 2.9 g/L, which was three-fold higher than that for its wild-type strain without the use of the resin (Yoon et al. 2007). Indeed, the utility of adsorbent resins with microporous structures can be observed from a study by Hua et al. (2007a, b) where vanillin produced by ferulic acid biotransformation by *Streptomyces* sp. strain V-1 was

adsorbed on the resins, leading to high production of 19.2 g/L along with ease of its downstream processing (Hua et al. 2007b). With a similar concept of obtaining the product adsorbed onto a resin (HD8), Zhao et al. (2006) also obtained decent production of vanillin (8.1 g/L) from isoeugenol. Recently, Luziatelli et al. (2019) reported production of approximately 4.9 g/L concentration of vanillin from ferulic acid in recombinant *E.coli* transformed with *fcs* and *ech* genes from a *Pseudomonas* strain, using the concept of resting cells, optimization of the bioprocess variables after using response surface methodology (RSM), and a unique solid-liquid separation system which had ferulic acid entrapped into 1.75% w/v agarose gel cylinders (Luziatelli et al. 2019). Here, in contrast to the product's adsorption, the substrate was immobilized for its steady release in the media. Overall, these studies largely demonstrate that product inhibition could be well sidestepped by the addition of adsorbent resins in the fermentation systems.

Finally, in terms of using genetically engineered strains as the hosts, the study by Yamada et al. (2007) is worth mentioning in which the authors cloned a rare isoeugenol monooxygenase gene from a *Pseudomonas putida* strain IE27 into *E. coli* BL21. With the expression of just a single gene in *E. coli*, the concentration of 28.3 g/L of vanillin was realized from 230 mM isoeugenol in 6 h (Yamada et al. 2008). The achieved concentration of 28.3 g/L was the highest concentrations of vanillin ever reported in the literature from the use of either recombinant or native cells or cell-free extracts. Although it was nearly close to the production attained using wild *Amycolatopsis* sp. 39116 (22.3 g/L) by Fleige et al. (2016). The use of *E. coli* as the host eliminates the complications associated with the use of *Amycolatopsis* like microorganisms that are spore formers and do not have the requisite Generally recognized As Safe (GRAS) status. Infact, Kaur et al. (2014) have reported heterologous expression of *fcs* and *ech* genes in a lactic acid bacterium, *Pediococcus acidilactici* BD16, with GRAS status. In their study, the authors could recover 3.14 mM of vanillin within 20 min from 1.08 mM ferulic acid (Kaur et al. 2014).

Nevertheless, the recombinant strains are associated with certain disadvantages such as their genetic instability, inappropriate genetic tools, and high cost associated with the cloning, transformation, or recombination. Hence, immobilization of vanillin-producing microbial cells as biofilms has gained attention as yet another strategy due to their exceptional operational stabilities when persistent bioconversion times are required, high cell concentrations, and tolerance against harsh environments. Yan et al. (2016) attempted biocatalysis of ferulic acid to vanillin in a packed bed bioreactor, which had *Bacillus subtilis* cells immobilized as biofilms on the carbon fiber textiles (CFT); their vanillin's reported production was 1.84 g/L, with the hydraulic retention time of just 20 h (Yan et al. 2016). Therefore, their process represents a faster production of vanillin in stable biotransformation where recycling or recovery of the immobilized biomass presents a potential economic advantage.

6.4.2 Specific Case of Polyhydroxyalkanoates (PHA) Production

Within the biopolymer group, polyhydroxyalkanoates or PHAs that are synthesized directly by microorganisms, are the plastic materials of the 21st century. PHAs are deposited intracellularly within the bacteria during the stress conditions, as energy storage or carbon reserves, (Getachew and Woldesenbet 2016). Their monomer building blocks, formed mainly from saturated or unsaturated hydroxy alkanoic acids, can vary in length from C3 to C14 carbon atoms with a variety of straight or branched chain aliphatic or aromatic side groups. Typically, the structure of PHAs depends on the feedstock monomers available together with the substrate specificity of the PHA synthase (PhaC).

There are reports available where microorganisms have been isolated in nature that grow on lignin as the sole carbon sources and transform derivatives of lignin to (*R*)-3-hydroxyacyl-CoA (3HA-CoA) via fatty acid de novo biosynthesis pathways for the biosynthesis of PHA. *Pandoraea* sp. ISTKB is one such strain tested for its ability to degrade lignin and use the released derivatives for PHA (Kumar et al. 2017). For this testing, the authors grew ISTKB on kraft lignin and its lignin derivatives, particularly syringol, vanillic acid, 4-hydroxybenzoic acid (4-HBA), *p*-coumaric acid, and 2,6-dimethoxyphenol (DMP), as the only carbon sources in the media, aerobically under nutrient-limited conditions for 6 days at 30 °C, pH 8, and 185 rpm. The concentration of PHA that *Pandoraea* sp. ISTKB accumulated was 246 mg/L with 4-HBA, followed by 170 mg/L with *p*-coumaric acid, 72 mg/L with *p*-coumaric acid, 69 mg/L with DMP, and 18 mg/L with kraft lignin (Kumar et al. 2017). Their results indicate that the bacterium's PHA accumulation decreased with an increase in the substrate's structural complexity. 4-HBA is an intermediate produced down the *p*-coumaric acid degradation pathway and seemingly had the simplest structure. Hence, ISTKB accumulated maximum PHA of the type poly (3-hydroxybutyric-co-hydroxyvaleric) acid *P*(*HB-co-HV*) with 4-HBA, and least with lignin (Kumar et al. 2017). Previously, with *Ralstonia eutropha* H16 as the model strain, Satoshi et al. (2014) reported similar results. The authors tested the capacity of H16 to synthesize *P* (*HB-co-HV*) from a variety of lignin derivatives. However, it was with 4-HBA and 3-HBA as the substrates that maximum PHA at 63 wt.% and 65 wt.% was accumulated by *R. eutropha* H16 (Tomizawa et al. 2014). With other intermediates, such as vanillic acid, ferulic acid, and *p*-coumaric acid, cell growth inhibition and PHA accumulation were observed (Tomizawa et al. 2014). Inhibition of cell growth was also observed in a γ-proteobacterium marine isolate *Oceanimonas doudoroffii* in the presence of p-coumaric acid, vanillic acid, ferulic acid, caffeic acid, and gallic acid. With *O. doudoroffii*, the authors reported reasonable PHA production (short-chain length polyhydroxyvalerate (PHV), 1.9 wt.% with sinapinic acid, and 2.7 wt.% with syringic acid (Numata and Morisaki 2015), which are again the simpler derivatives produced during lignin degradation.

In contrast to the aforementioned observations, where microorganisms tend to be inhibited by lignin, strains of the genus *Pseudomonas* have been reported for PHA

production from lignin itself. For instance, *P. putida* KT2440 has been demonstrated to produce 150 mg/L medium chain-length (C6-C14) polyhydroxyalkanoates (*mcl*-PHAs) from alkaline pretreated corn liquor rich in lignin (32% wt./wt.) and extractives of lignin (23% wt./wt. of *p*-coumaric acid, vanillic acid, and ferulic acid) by Linger et al. (2014). The *mcl*-PHA biopolymer produced by *P. putida* KT2440 has a molecular weight of 124 kDa and has side chains comprising 3-hydroxydecanoic acid (55%), 3-hydroxyoctanoic acid (22%), 3-hydroxydodecanoic acid (16%), 3-hydroxytetradecancoic acid (4%), and 3-hydroxyhexanoic acid (3%) (Linger et al. 2014). Alkaline pretreated lignin (APL) from corn stover was also used to support *P. putida* KT2440, *P. putida* mt-2, and *Cupriavidus necato* growth and PHA accumulation (Salvachúa et al. 2015). Therein, the *P. putida* KT2440 and *P. putida* mt-2 synthesized 52 mg/L and 60 mg/L of mcl-PHA, respectively. *C. necator* was found to accumulate 162 mg/L of short-chain length polyhydroxy butyrate (PHB) (Salvachúa et al. 2015). More recently, *Pandoraea* sp. B-6 has also been shown to carry the potential to bio convert kraft lignin into PHA. Strain B-6 was shown to degrade 40% of kraft lignin in barely 4 days, with a resultant 24.7% accumulation of scl-PHB (Liu et al. 2019a). Similar findings were reported by Shi et al. (2017) with *Cupriavidus basilensis* B-8, which could accumulate 128 mg/L of PHB from kraft lignin (without any pretreatment) as the sole carbon source in 7 days (Shi et al. 2017). The authors in the study also highlighted the utility of fed-batch in enhancing PHB production during the bioconversion, as 319.4 mg/L of PHB was reported with 5 g/L of lignin using the fed-batch mode of fermentation (Shi et al. 2017).

Overall, these studies lay a solid foundation for pursuing bioconversion of lignin-rich streams to value-added biopolymers (Fig. 6.2). Generally, lignin and its aromatic intermediates are recalcitrant and toxic, and have been found to impede the fermentation and bioconversions in the host. In the future, optimization of culture conditions, the use of innovative fermentation modes, isolating new microbial strains, and metabolically engineering existing strains may prove useful for enhancing the yield and percent accumulation of PHA inside the host when lignin or its intermediates are used as the carbon source. Recently, CRISPR/Cas9n-based tool was used to engineer *Pseudomonas putida* KT2440 to produce a higher amount of mcl-PHA (270 mg/L) using ferulic acid as the feedstock. In yet another bioengineering study, Lin et al. (2016) improved the tolerance and productivity of *Pseudomonas putida* strain A514 toward lignin and its derivative vanillin by overexpressing a gene that codes for the VanAB enzyme of the β-ketoadipate pathway that is explicitly induced in the presence of vanillic acid (Lin et al. 2016). The group further channelized the vanillin bioconversion towards mcl-PHA synthesis by overexpressing phaB and phaC genes in A514. The results indicated that the modified A514 could accumulate 65 mg/L PHA, with a yield of 73.5% per CDW compared with 54% in the wild strain of A514. After this two-step metabolic engineering, A514 was also able to accumulate 75 mg/L mcl PHA (C8-C14) with kraft lignin, which is significantly more recalcitrant than the processed APL lignin (Lin et al. 2016). In addition, the authors reported that enhanced PHA production occurred through the complete growth cycle for both nitrogen-limiting and nitrogen-excess conditions. Moreover, the composition of the produced mcl-PHA

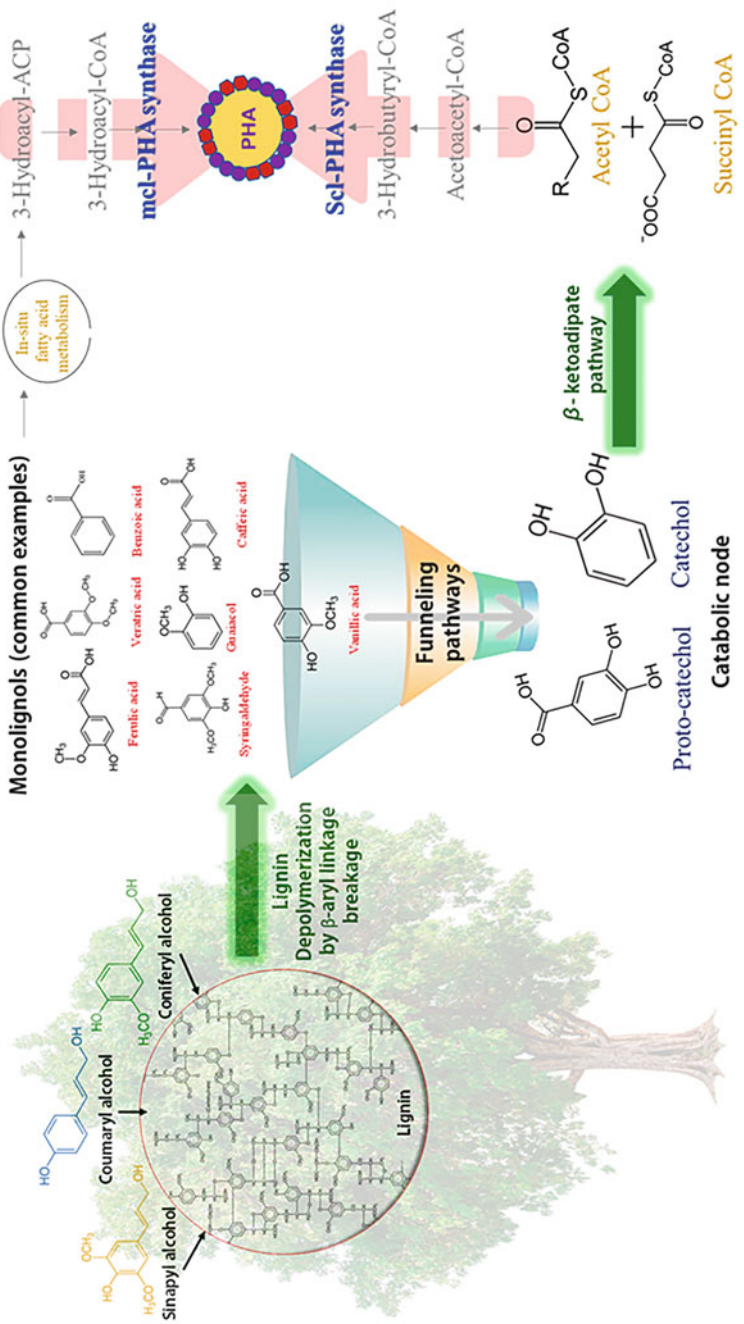

Fig. 6.2 General scheme for bioconversion of lignin-rich streams into polyhydroxyalkanoates

had side chains ranging from C8-C14, which in itself is valued highly for its ability to be a precursor for the synthesis of jet fuels (C8-C16) (Lin et al. 2016). Wang et al. (2018) enhanced the PHA production of *Pseudomonas putida* strain A514 to 246 mg/L from vanillin by further overexpression of long-chain fatty acid-CoA ligase (*alkK*), and 3-hydroxyacyl-acyl carrier protein (ACP) thioesterase (*phaG*). In the same study, these genes were identified after an extensive study of genomics, transcriptomics, and proteomics data of A514 grown on vanillin under nitrogen-limited conditions (Wang et al. 2018). Thus, molecular insights based on omics study can help identify schemes to enhance PHA production from lignin or its derivatives.

Generally, for synthesizing high-molecular-weight PHA copolymers, expensive odd fatty acids (propionic acid, valeric acid) are supplemented in the medium as co-carbon substrates (Govil et al. 2020b). This co-addition of additional feedstocks not only increases the cost of production of PHA copolymer but also has a low yield and accumulation of copolymers compared to the addition of homopolymers. However, the studies discussed in this section suggest that the lignin or its aromatic derivatives can serve as low-cost platform precursors for synthesizing not only scl-copolymers like P(HV) and *P*(*HB-co-HV*) but also mcl-PHAs with side chains ranging from 3-hydroxyhexanoic acid to hydroxy-tetra decanoate.

Currently, the valorization process of lignin to PHA has an important drawback. The concentration and the yield of the produced PHA are low, being in the tens or low hundreds of milligrams per liter. The principal reason for this low yield is related to the low reactivity and assimilation of lignin by the microbial hosts. However, a study published in 2019 showed that co-utilization of lignin with a limited amount of glucose could facilitate lignin biocatalysis to PHA. The authors used this concept to produce the record titer of 1.5 g/L of PHA by the synergistic bioconversion of lignin and residual sugar released during corn stover pretreatment and hydrolysis by the *Pseudomonas putida* strain KT2440 (Liu et al. 2019b). This shows that lignin-based biorefinery sustainability is conceivable by applying innovative yet straightforward concepts.

6.5 Conclusion and Future Directions

Lignin, a polyaromatic macromolecule, is one of the most underutilized fractions of the lignocellulosic biomasses, whose valorization to fine chemicals has a much higher economic and environmental benefit than burning it for heat and electricity. The abundance of aromatic monomers in its skeletal structure makes lignin a promising substrate for biocatalysis into an array of value-added products such as aromatic biomolecules, biopolymers, bio-oil, and biofuels. Today, lignin is also considered a commendable, environmental-friendly component or additive in the preparation of epoxy resins, fire-retardants, antioxidants, adhesives, and concrete admixtures. Hence, the production of lignin-derived co-products can support, and enhance the profitability of second-generation biorefineries, and related industries.

With the advancement of multi-omics knowledge, the sophisticated metabolic pathways essential for lignin degradation are being elucidated in detail. In the future, engineering of these pathways in microbes for the overproduction of useful intermediates such as ferulic acid, vanillin, and guaiacol is foreseen. In addition, the central target for the near future should be the improvement of the technology for separating lignin efficiently and cost-effectively from lignocellulosic biomass.

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