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Lignin valorization through microbial production of polyhydroxyalkanoates: recent trends, challenges and opportunities

Zhe Liang,[a,b] Sivasamy Sethupathy, *[a,b] Dang Wenqian,[a,b] Hu Jinhao [a,b] and Daochen Zhu *[a,b]

Polyhydroxyalkanoates (PHA) are biopolymers produced by bacteria under nitrogen-limited and carbon-rich conditions and have been explored as a potential replacement for petroleum-based plastics. Despite their versatile applications and non-toxic and eco-friendly properties, PHAs currently hold a relatively low market share owing to their high production and downstream processing costs. However, lignin, a renewable aromatic source and byproduct of lignocellulose biorefineries, is considered a cheap substrate for microbial production of PHA. Research over the past decade has demonstrated that microbes with diverse aromatic metabolic pathways can degrade lignin and effectively transform lignin-derived aromatic compounds (LDACs) into PHA by biofuneling them into their central carbon metabolism. Recent advances in lignin extraction, lignin depolymerisation, genetic, metabolic, and protein engineering, multiomics approaches, artificial intelligence, and development of efficient fermentation and downstream processing methods have paved the way for sustainable production of PHA from lignin. In light of these developments, this review comprehensively examines the metabolic pathways involved in the utilisation of LDACs and recent developments in improving microbial production of PHA. We also discuss the challenges and opportunities to improve several aspects of the bioconversion of lignin into PHA, from the perspectives of both lignin and bacterial processes.

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1. Recent advancements in the production of polyhydroxyalkanoates (PHA) from lignin and lignin-derived aromatic compounds offer a sustainable alternative to traditional petroleum-based plastics.
2. This review presents a comprehensive overview of genetic, metabolic, and protein engineering approaches for enhancing PHA production using industrially important bacterial strains over the last decade. Furthermore, this review addresses the current challenges and explores potential opportunities for improving PHA production.
3. In addition, this review briefly discusses advancements in lignin depolymerization techniques, which are crucial for optimizing PHA production. By examining these aspects, this review aims to provide insights and suggest future research directions for PHA production from lignin.

1. Introduction

As a consequence of the emergence of a series of challenges associated with energy shortages, the depletion of resources and environmental pollution has led to a notable increase in focus towards the exploration of clean and renewable energy sources. Lignin has attracted considerable interest from the scientific community as a renewable and cost-effective potential energy source.[1–3] Plant cell walls are composed of cellulose, hemicellulose and lignin, with a lignin content of ~15–30% and an energy content of ~40%.[4] The global annual production of lignin is estimated to reach up to 10 billion tons.[5] Lignin is a complex aromatic polymer consisting of three monolignols with a phenylpropane structure, namely paracoumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S).[6] Lignin multimers are formed by the interconnection of multiple monolignols, which exist in varying proportions in different plants.[7] Depending on the proportion of monolignols, lignin can be classified into three types: H-type (*p*-hydroxyphenyl), G-type (guaiacyl) and S-type (syringyl). In addition to these three primary monolignols, plants produce

[a]*International Joint Laboratory on Synthetic Biology and Biomass Biorefinery, Biofuels Institute, School of the Environment and Safety Engineering, Jiangsu University, Zhenjiang 212013, China. E-mail: sethulifescience@gmail.com, dczhucn@ujs.edu.cn*
[b]*Jiangsu Collaborative Innovation Center of Technology and Material of Water Treatment, Suzhou University of Science and Technology, Suzhou 215009, China*

various non-conventional lignin monomers. These non-conventional monomers are derived from canonical monolignols, flavonoids, hydroxystilbenes and hydroxycinnamamides, such as monolignol acetates, tricin, piceatannol, and diferuloylputrescine, respectively and are incorporated into lignin structures.[8,9] It is possible that in the future the composition of lignin can be modified through genetic engineering of bioenergy crops. This could potentially broaden the range of lignin derived high value products and ease up the lignin depolymerization.[10–12]

Despite extensive research on lignin depolymerization, its complete utilization remains a herculean task; thus, it is invariably utilized for power generation through combustion[13,14] and there is a pressing need to convert lignin into valuable products in the future.[15] In recent years, there have been significant developments in lignin depolymerization using various chemical, physical, and biological methods. By combining these methods, aromatic monomer production from lignin can be improved. As research and development in lignin valorization continues to progress, the focus remains on the complete depolymerization of lignin to harness its full economic potential and improve the selectivity of aromatic monomer production.

A significant issue associated with petroleum-based plastics is their propensity to generate non-degradable waste during their operational lifespan,[16] which has the potential to exert a considerable environmental burden. Biopolymer polyhydroxyalkanoates (PHAs) are derived from renewable and biological sources and typically display properties analogous to those of petroleum-based polymers.[17] It can be biodegraded into non-toxic and harmless byproducts. A plethora of microorganisms in nature have evolved sophisticated enzymatic catabolic systems and metabolic pathways for lignin over long periods of time.[18] The production of PHA by microbial action using abundant lignin as a raw material represents a promising avenue of research in recent years.[19] This attention stems from several key factors, such as the abundance and renewable nature of lignin, the potential to create additional revenue streams for lignocellulosic biorefineries, and PHA as an alternative to conventional plastics. This approach not only addresses the growing demand for eco-friendly materials, but also offers a valuable solution for valorising lignin. Currently, PHA production from lignin is yet to be industrialised due to high production and downstream processing costs. Nevertheless, this approach is widely regarded as sustainable, offering a low carbon footprint that aligns well with the principles of a circular economy. Significant efforts to reduce the overall production cost have shown promise, with advancements in lignin fractionation from biomass, its depolymerisation to produce fermentable aromatic compounds, and genetic and metabolic engineering of microbes to enhance PHA yields. The growing awareness of the use of biodegradable plastics, coupled with regulatory measures on plastic waste disposal and government incentives for the production of bioplastics using renewable feedstocks, is expected to drive market demand and acceptance of PHA. Furthermore, increased collaboration between academia and industry is likely to accelerate PHA production from lignin and commercialisation. Considering the importance of maximum utilisation of lignin and applications of PHA, in this review, we comprehensively reviewed the recent developments, challenges, and opportunities in lignin depolymerisation, PHA production from LDACs, and downstream processing.

2. Polyhydroxyalkanoates: brief introduction

As the global population expands and the global economy experiences sustained growth, the annual production of plastics continues to increase.[20] The uncontrolled use of plastics has caused serious environmental pollution. When discarded plastics enter our ecosystem, they are not decomposed but instead break into smaller particles that remain in the soil and enter the water as rivers converge in the ocean, creating a 'plastic soup' of pollution.[21] To address this issue effectively, it is crucial to focus on the production of environmentally friendly and sustainable alternatives, such as bioplastics and biodegradable plastics. The biodegradability and environmental friendliness of bioplastics have generated considerable interest, with research focusing on a range of materials, including PHA, polylactic acid (PLA), poly(ε-caprolactone) (PCL), polyethylene, and poly(ester amide)s.[22]

PHA is a linear polyester polymer synthesised and accumulated intracellularly as a carbon and energy reserve by bacteria under specific nutritional conditions, mainly in the presence of abundant carbon sources and a lack of nitrogen and phosphorus sources.[23,24] Based on the number of carbon atoms in the monomer, PHA can be classified into three main categories:[25] Short-chain-length PHA (scl-PHA), in which the monomer unit comprises three to five carbon atoms, as exemplified by poly(3-hydroxyvaleric acid) (PHV) and poly(3-hydroxybutyric acid) (PHB);[26] Medium-chain-length PHA (mcl-PHA), in which poly(3-hydroxyoctanoate) (PHO) is a polyester comprising 6–16 carbon atoms per monomer; and a combination of short and medium-long chains to form a copolymer PH[27,28] (Fig. 1). Furthermore, under specific circumstances, two or more monomers can be connected by ester bonds to create copolymers such as 3-hydroxybutyric acid (3HB) and 3-hydroxyhexanoic acid (3HHx), among others. PHA possess a diverse range of monomer types and structural characteristics.[29] Therefore, they exhibit different characteristics.[30]

Furthermore, biocompatibility, non-toxicity, and non-immunogenic properties of PHA present promising applications in the biopharmaceutical industry.[31] Notably, biocompatibility, bioactivity and biodegradability of PHA have been tested under *in vitro* and *in vivo* conditions.[32] In addition, researchers have shown that PHA membranes favor high adhesion of epithelial and osteoblast cells.[33] Furthermore, PHA films have been used as wound dressing materials.[34] Upon entering the bloodstream, PHA does not induce an immune response and exhibits good hemocompatibility; thus,

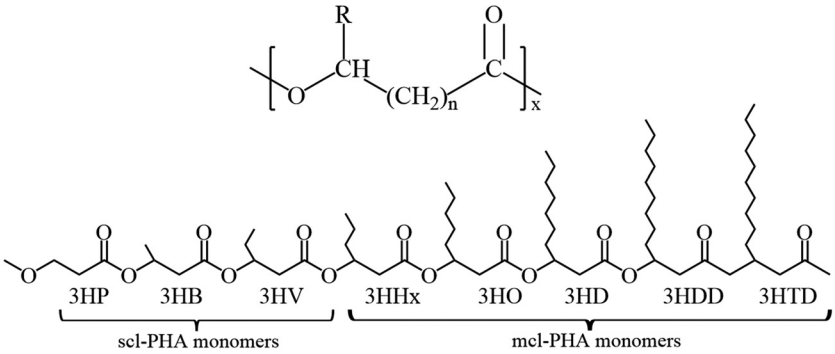

Fig. 1 Generic structure of PHA and structure of scl and mcl-PHA monomers. *n* = 1 to 4; R = alkyl group or other functionalized groups (3HP, 3-hydroxypropoinate; 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctonoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate; 3HTD, 3-hydroxytetradecanoate).

scaffolds made of PHA allow cells to grow through and have sufficient supportive properties in the vasculature,[35] to act as scaffolds that promote cell growth.[36] In the pharmaceutical field, PHAs are used in the synthesis of nanoparticles for the controlled and targeted delivery of drugs.[37] PHA can be modified at the structural level through the application of physical, chemical and biological processes, resulting in the production of a novel polymer with predictable alterations in functionality and molecular weight. This enables the creation of PHA materials that are better suited to the requirements of specific applications and that can serve as a more sustainable alternative to traditional petroleum-based plastics.

2.1. Pathways for conversion of LDACs into PHA

Enzymatic and non-enzymatic depolymerisation of lignin yields various aromatic compounds (ferulic acid, vanillin, vanillic acid, syringaldehyde, guaiacol, *p*-hydroxybenzoic acid, *etc*.), dimeric compounds, and low molecular weight fragments[3,38] (Fig. 2). Despite the abundance of microorganisms, only a few are capable of transforming these aromatic compounds through their metabolic pathways, such as CoA-dependent β-oxidation, CoA-dependent non-β-oxidation, non-oxidative decarboxylation, and side chain reduction, into key aromatic metabolic intermediates. These aromatic metabolic intermediates include vanillic acid, protocatechuic acid (PCA), and catechol. This is followed by the conversion of PCA and catechol into central metabolic intermediates (acetyl-CoA and succinyl-CoA) through aromatic ring-opening pathways such as 2,3-cleavage pathway, 3,4-cleavage pathway and 4,5-cleavage pathway for PCA and *ortho*-cleavage pathway for catechol[19] (Fig. 3). Subsequently, the central metabolic intermediates are transformed into commercially valuable products, including PHA, triacyl glyceride, lactic acid, lactones, terpenes, and alkanes.

Microbes utilize non-oxidative decarboxylation pathway, CoA-independent pathway and CoA-dependent oxidation pathways (which can be further divided into non-β-oxidation and β-oxidation pathways) to metabolise H-lignin derivatives, exemplified by *p*-coumaric acid.[39,40] Through these pathways, *p*-coumaric acid undergoes a series of transformations catalysed by monooxygenases, reductases, and dioxygenases to produce PCA (Fig. 3).[41,42] These complex and multifaceted metabolic pathways indicate microbial evolutionary adaptations to utilise LDACs as carbon sources.

Ferulic acid, a representative G-lignin derivative, carries a methoxy group on its benzene ring. Ferulic acid can be converted to vanillic acid *via* four pathways: non-oxidative decarboxylation pathway, CoA-dependent oxidation pathway (non-β-oxidation pathway and β-oxidation pathway), and side chain reduction pathway (Fig. 3),[43] and the methoxy group on the benzene ring of vanillic acid is converted into a hydroxyl group by demethylase to produce protocatechuic acid. Briefly, ferulic acid can be converted into PCA *via* CoA-dependent β-oxidation, CoA-dependent non-β-oxidation, CoA-dependent oxidation, and non-oxidative decarboxylation pathways, with vanillin as a central aromatic metabolite. The CoA-dependent β-oxidation pathway activates ferulic acid into its CoA thioester (feruloyl-CoA) by feruloyl-CoA synthetase, followed by hydration, β-oxidation, and thioclastic cleavage to produce vanillin (Fig. 3). The non-β-oxidation pathway involves the transformation of ferulic acid to feruloyl-CoA into vanillin with 4-hydroxy-3-methoxy-β-hydroxypropinoyl-CoA as an intermediate, for example in *Streptomyces* sp. Strain V-1.[44] The non-oxidative decarboxylation pathway transforms ferulic acid into 4-vinylguaiacol *via* ferulic acid decarboxylase (PAD) and then into vanillin by carotenoid cleavage oxygenase (Cso2) or vinyl guaiacol dehydrogenase (VGDH). In contrast, the side chain reduction pathway involves the conversion of ferulic acid into dihydroferulic acid by aromatic reductases, which is converted to vanillin by decarboxylases. Subsequently, vanillin is converted to vanillic acid by vanillin dehydrogenase and to PCA (Fig. 3).

Notably, vanillin synthase (VpVAN), found in Vanilla planifolia, can directly transform ferulic acid into vanillin. This biotransformation occurs through hydratase/lyase activity, without requiring any cofactors such as NAD^+ or ATP.[45] A recent study showed that an engineered coenzyme-independent dioxygenase Ado (F82Y/V332R/F334R), derived from *Thermothelomyces thermophilus* ATCC 42464 can transform

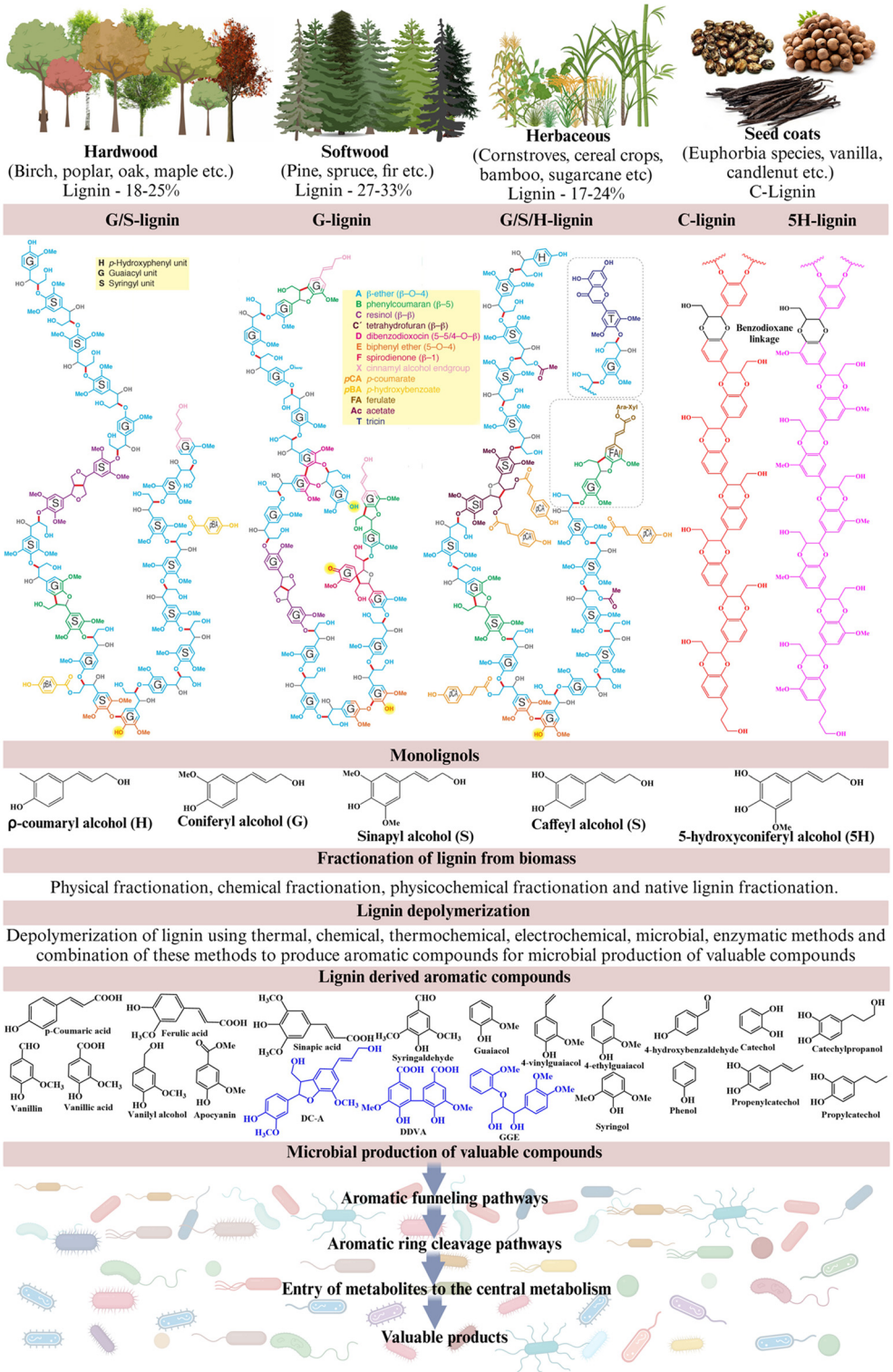

Fig. 2 Major sources of lignin, representative structures of G/S, G, G/S/H, C, and 5H lignin and their monolignols. Overview of lignin fractionation, lignin depolymerization, lignin-derived aromatic compounds, and microbial transformation. Structures of lignin are reproduced from Ralph *et al.* (2019)[222] and Wang *et al.* (2022)[223] with permission. Created using BioRender (https://BioRender.com).

ferulic acid, coniferyl alcohol into vanillin. Additionally, this engineered enzyme can transform *p*-coumaric acid, sinapinic acid into *p*-hydroxybenzaldehyde and syringaldehyde.[46] Future studies should aim to identify these types of distinctive enzymes from available microbial whole-genome and metagenomic datasets. This, coupled with enzyme structural analysis

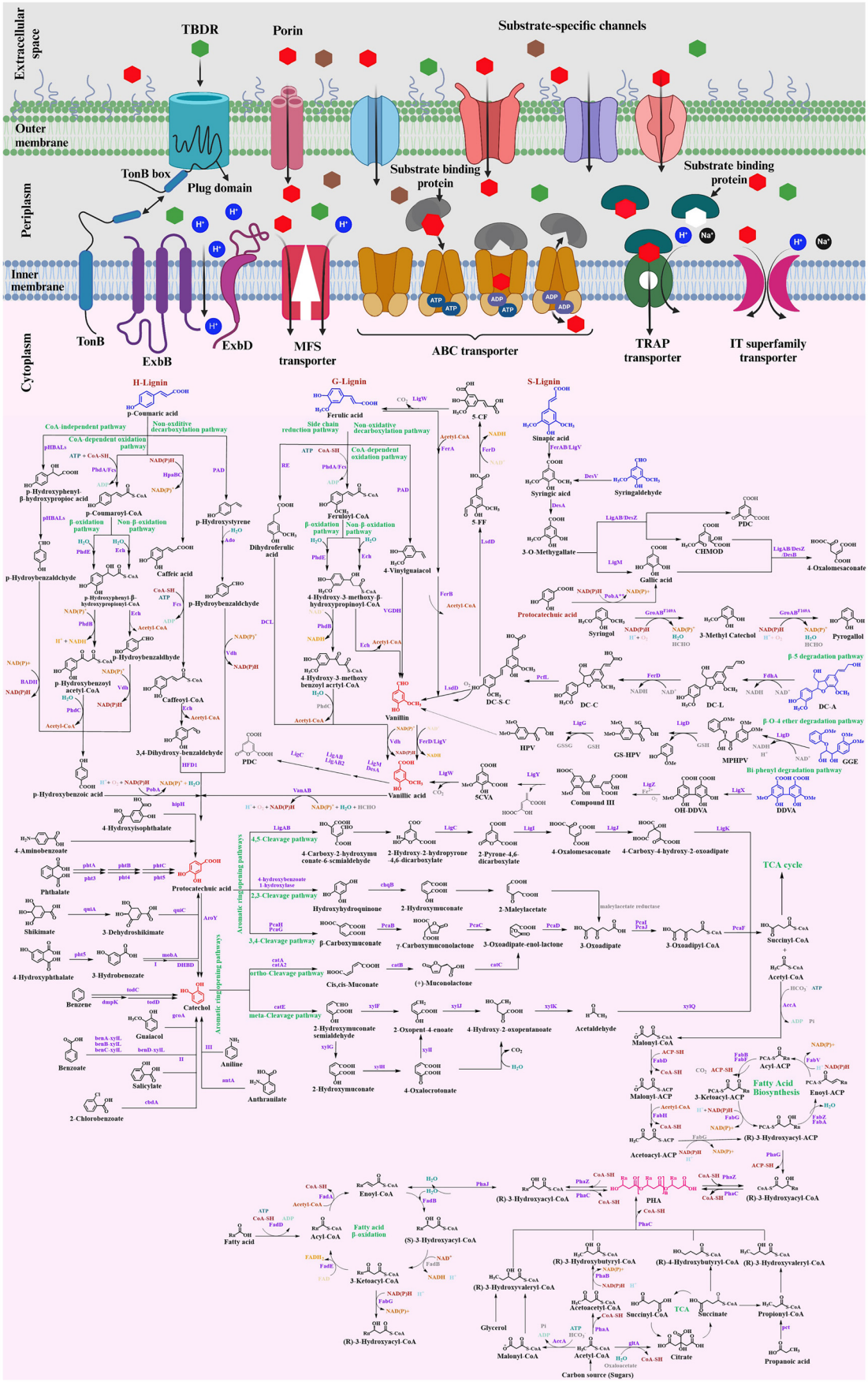

Fig. 3 *(Contd)*.

Fig. 3 Overview of transporter proteins/systems involved in the uptake of lignin-derived aromatic compounds in bacteria (inspired and modified from Kamimura *et al.*, 2017[224]) and biofuneling pathways for metabolising lignin-derived aromatic compounds to produce PHA (inspired and modified from Weng *et al.*, 2021;[40] Salvachúa *et al.*, 2020;[113] de Gonzalo *et al.*, 2016;[225] Liu *et al.*, 2022;[226] Liu *et al.*, 2022[227] and Metz *et al.*, 2024[228]). Created using BioRender (https://BioRender.com) and kingDraw (https://kingdraw.cn/). AccA, acetyl-CoA carboxylase; Ado, aromatic dioxygenase; antA, anthranilate 1,2-dioxygenase; AroY, protocatechuate decarboxylase; BADH, benzaldehyde dehydrogenase; catA/catA2, catechol 1,2-dioxygenase; catB/catC, muconate cycloisomerase; catE, catechol 2,3-dioxygenase; cbdA, 2-halobenzoate 1,2-dioxygenase large subunit; CHMOD, 4-carboxy-2-hydroxy-6-methoxy-6-oxohexa-2,4-dienoate; chqB, hydroxyquinol 1,2-dioxygenase; DC-A, dehydrodiconiferyl alcohol; DC-C, dehydrodiconiferyl carboxylic acid; DC-L, dehydrodiconiferyl aldehyde; DCL, decarboxylase; DC-S-C, dehydrodiconiferyl stilbene carboxylic acid; DDVA, 5, 5′-dehydrodivanillate; DesA, *O*-demethylase; DesV, aromatic aldehyde dehydrogenase; DHBD, 2,3-dihydroxybenzoate decarboxylase; dmpK, phenol/toluene 2-monooxygenase; Ech, enoyl-CoA hydratase; Fcs, acyl-CoA synthetase; FabA and FabZ, 3-hydroxyacyl-ACP dehydratase; FabB and FabF, 3-oxoacyl-ACP synthase; FabD, malonyl-CoA-ACP transacylase; FabG, 3-ketoacyl-ACP reductase; FabH, 3-ketoacyl-ACP synthase; FabV, enoyl-ACP reductase; FadA, 3-ketoacyl-CoA thiolase; FadB, 3-hydroxyacyl-CoA dehydrogenase; FadD, long-chain acyl-CoA synthetase; FadE, acyl-CoA dehydrogenase; FdhA, glutathione-independent formaldehyde dehydrogenase; FerA, feruloyl-CoA synthetase; FerB, feruloyl-CoA hydratase/lyase; FerD, aldehyde dehydrogenase; gcoA, aromatic *O*-demethylase, cytochrome P450 subunit; GGE, guaiacylglycerol-β-guaiacylethe; GroAB, cytochrome P450; GS-HPV, α-glutathionyl-HPV; GroABF169A, the mutation of GroAB; hipH, 4-hydroxyisophthalate hydroxylase; HFD1, hexadecenal dehydrogenase; HpaBC, 4-hydroxyphenylacetate 3-monooxygenase oxygenase; HPV, β-hydroxyproppiovanillone; LigAB, aromatic-ring-opening dioxygenase; LigC, 4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase; LigD, bifunctional non-homologous end joining protein; LigG, glutathione S-transferase; LigI, 2-pyrone-4,6-dicarboxylate hydrolase; LigJ, 4-oxalomesaconate hydratase; LigM, 3-*O*-methygallate *O*-demethylase; LigV, vanillin dehydrogenase; LigK, 4-carboxy-4-hydroxy-2-oxoadipate aldolase; LigW, 5-carboxyvanillate decarboxylase; LigX, DDVA *O*-demethylase; LigY, OH-DDVA *meta*-cleavage compound hydrolase; LigZ; OH-DDVA oxygenase; mobA, 3-hydroxybenzoate 4-monooxygenase; LsdD, lignostilbene dioxygenase; MPHPV, α-(2-methoxyphenoxy)-β-hydroxypropiovanillone; OH-DDVA, 2,2′-3-trihydroxy-3′-methoxy-5,5′-dicarboxybiphenyl; PAD, ferulic acid decarboxylase; PcaHG, protocatechuate 3,4-dioxygenase; PcaB, 3-carboxy-*cis*,*cis*-muconate cycloisomerase; PcaC, 4-carboxymuconolactone decarboxylase; PcaD, 3-oxoadipate enol-lactonase; PcaI/PcaJ, 3-oxoadipate CoA-transferase; PcaF, 4-hydroxy-2-oxovalerate aldolase; PcfL, γ-formaldehyde lyase; PDC, 2-pyrone-4,6-dicarboxylate; PhaA, beta-ketothiolase; PhaB, Acetoacetyl CoA reductase; pct, propionate CoA-transferase; PhaC, poly[(*R*)-3-hydroxyalkanoate] polymerase; PhaG, hydroxyacyl-ACP acyl-transferase; PhaJ, (*R*)-enoyl-CoA hydratase; gltA, citrate synthase; PhaZ, PHA depolymerase; pHBALS, *p*-hydroxybenzaldehydesynthase; PhdA, *p*-hydroxycinnamoyl-CoA synthetase; PhdB, 3-hydroxyacyl-CoA dehydrogenase; PhdC, 3-oxoacyl-CoA ketohydrolase; pht3, phthalate 4,5-dioxygenase; pht4, phthalate 4,5-*cis*-dihydrodiol dehydrogenase; pht5, 4,5-dihydroxyphthalate decarboxylase; phtA, phthalate 3,4-dioxygenase; phtB, phthalate 3,4-*cis*-dihydrodiol dehydrogenase; phtC, 3,4-dihydroxyphthalate decarboxylase; PobA, *p*-hydroxybenzoate hydroxylase; quiA, quinate dehydrogenase; PobA**, the mutant of *p*-hydroxybenzoate hydroxylase; quiC, 3-dehydroshikimate dehydratase; todC1, benzene/toluene/chlorobenzene dioxygenase subunit alpha; todD, *cis*-1,2-dihydrobenzene-1,2-diol dehydrogenase; VanAB, vanillate *O*-demethylase oxygenase; Vdh, vanillin dehydrogenase; RE, aromatic reductase; VGDH, vinyl guaiacol dehydrogenase; xylF, 2-hydroxymuconate-semialdehyde hydrolase; xylG, aminomuconate-semialdehyde dehydrogenase; xylH, 4-oxalocrotonate tautomerase; xylI, 2-oxo-3-hexenedioate decarboxylase; xylJ, 2-oxopent-4-enoate hydratase; xylK, 4-hydroxy-2-oxovalerate aldolase; xylQ, acetaldehyde/propanal dehydrogenase; I, 3-hydroxybenzoate 2-monooxygenase; II, salicylate hydroxylase; III, aniline dioxygenase; 5-CF, 5-carboxyferulate; 5CVA, 5-carboxyvanillic acid; 5-FF, 5-formylferulate.

and engineering, will contribute the development of efficient ways to transform LDACs into vanillin and other valuable metabolites.

Sinapic acid, a derivative of S-lignin, contains two methoxyl groups, which makes it more challenging to break down and utilize than ferulic acid and *p*-coumaric acid. Moreover, only a few microorganisms can metabolize S-lignin derivatives. In *Sphingomonas paucimobilis* SYK-6, vanillin dehydrogenase (LigV) facilitates the transformation of sinapic acid into syringic acid (Fig. 3). Subsequently, syringic acid undergoes degradation *via* three distinct pathways: 1. syringate *O*-demethylase (DesA) removes a methyl group from syringic acid to produce 3-*O*-methyl gallate (3MGA), followed by conversion of 3MGA to 2-pyrone-4,6-dicarboxylate (PDC) by 3MGA 3,4-dioxygenase (DesZ). 2. DesZ and a hydrolase convert 3MGA into 4-oxalomesaconate (OMA) *via* the intermediate 4-carboxy-2-hydroxy-6-methoxy-6-oxohexa-2,4-dienoate (CHMOD). 3. *O*-Demethylase transforms 3MGA into gallate, which is then converted to OMA by gallate dioxygenase. Finally, OMA hydratase (LigJ) and 4-carboxy-4-hydroxy-2-oxoadipate aldolase/oxaloacetate decarboxylase (LigK) convert the OMA into two pyruvate molecules. These pyruvate molecules then enter the tricarboxylic acid (TCA) cycle. Our previous study showed that *Comamonas serinivorans* SP-35 has a metabolic pathway that transforms syringaldehyde into gallate, with syringate and 3-*O*-methylgallate as intermediates, through aldehyde dehydrogenases and *O*-demethylase oxidoreductases.[47] In addition, *Burkholderia* sp. ISTR5 produces malic acid from syringaldehyde, indicating the presence of a degradation pathway.[48,49] The abundance of syringyl units (≈60%) in lignin derived from poplar and corn necessitates isolation of microbes capable of degrading S-lignin derivatives is crucial for advancing lignin valorization and enhancing overall biomass utilization.

Lignin depolymerization either by biological, chemical, physical or a combination of these methods yields not only standard monomers but also a diverse range of aromatic compounds, presenting challenges in lignin valorization.[50] Thus, bacterial strains employ complex enzymatic pathways to transform a mixture of LDACs into useful metabolic intermediates. However, the metabolic capabilities of a bacterial strain are insufficient to metabolize these aromatic compounds. To address this bottleneck, metabolic engineering approaches have been developed to expand the metabolic repertoire of target bacteria. By heterologously expressing necessary genes from other organisms, in recent years researchers have developed highly efficient bacterial strains of *P. putida* KT2440 and *Halomonas* sp. for lignin valorization. This promising strategy enables the maximum utilization of LDACs on an industrial scale and potentially offers economic viability for lignin valorization.

2.2. Entry of key metabolic intermediates into the PHA synthesis pathway

The metabolism of LDACs results in the production of principal intermediates such as PCA and catechol (Fig. 3). These intermediates can enter diverse metabolic pathways and are ultimately converted into succinyl coenzyme A and acetyl coenzyme A.[108] Briefly, PCA undergoes ring opening *via* 2,3-cleavage (2,3-*meta*-cleavage), 3,4-cleavage (β-ketoadipate or *ortho* cleavage), and 4,5-cleavage (4,5-*meta*-cleavage) pathways. The 2,3-cleavage pathway produces pyruvate and acetyl-CoA, the 4,5-cleavage pathway produces two pyruvate molecules, and the 3,4-cleavage pathway produces succinate and acetyl-CoA. Catechol, another important intermediate, enters pathways similar to PCA, specifically the 2,3-cleavage and otho-cleavage pathways, and gives rise to pyruvate and acetyl-CoA, and succinate and acetyl-CoA, respectively (Fig. 3).

Bacteria utilize various carbon sources, including glucose, sucrose, lactose, and LDACs, and convert them to acetyl-CoA through three metabolic pathways (Fig. 3). Briefly, in pathway I, sugars undergo glycolysis to produce acetyl-CoA, followed by a thioester-dependent Claisen condensation reaction, in which two acetyl-CoA molecules are condensed by acetoacetyl-CoA thiolase (phaA) to form acetoacetyl-CoA. Subsequently, acetoacetyl-CoA is reduced to (*R*)-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (PhaB) and transformed into scl-PHA by PHA synthase (PhaC). In pathway II, the β-oxidation of fatty acids produces 2-*trans*-enoyl-CoA and 3-ketoacyl-CoA, which are then transformed into (*R*)-3-hydroxyacyl-CoA by R-specific enoyl-CoA hydratase (PhaJ) and 3-ketoacyl-CoA reductase (FabG), respectively, which are subsequently used for PHA production. In pathway III, acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (AccA-D), followed by the production of malonyl-ACP and acetoacyl-ACP by malonyl CoA-ACP transacylase (FabD) and 3-ketoacyl-ACP synthase (FabH), respectively. Finally, acetoacyl-ACP enters the fatty acid biosynthesis pathway to generate (*R*)-3-hydroxyacyl-ACP, which is converted to (*R*)-3-hydroxyacyl-CoA by 3-ketoacyl-ACP reductase (FabG). (*R*)-3-Hydroxyacyl-ACP is subsequently utilized by PHA polymerases (PhaC) for PHA synthesis (Fig. 3).

3. Challenges for lignin bioconversion to PHA

Transforming lignin into PHA involves a series of steps, each presenting its own set of challenges.

3.1. Fractionation of lignin from biomass

Because lignin is intertwined with cellulose and hemicellulose in plant cell walls, delignification requires extreme pretreatment conditions involving high temperatures, pressures, and chemical treatments. Achieving high lignin fractionation, uniform lignin quality, and preservation of cellulose quality across diverse batches and types of lignocellulose biomass is crucial for industries. Widely used isolation methods in biorefineries induce structural changes, and the condensation of lignin makes it more recalcitrant to depolymerization to produce aromatic compounds. The microbial conversion of lignin into valuable products requires a separation technique which minimizes modifications, condensation, and solubility issues. Thus, extensive research has focused on developing simple extraction methods that balance the ecofriendliness, extraction efficiency, retention of the native structure of lignin, and its applicability to various biomass sources. The lignin-first biorefinery approach is aimed at the selective extraction of lignin and its valorization before processing the cellulose and hemicellulose fractions. This process utilizes solvolysis or reductive catalytic fractionation (RCF) to extract lignin from biomass with minimal structural modification and condensation.[51] The delignification and depolymerisation of lignin from milled beech wood by manganese peroxidase (MnP) and lignin peroxidase (LiP) in batch and membrane bioreactors produced aromatic compounds and low-molecular-weight lignin. Interestingly, when compared to depolymerisation in batch bioreactors, depolymerisation in membrane bioreactors using MnP and LiP produced more low-molecular-weight lignin, which can be readily used for further valorization using microbes.[52] Recent developments in computational and machine learning tools, including the COnductor-like Screening MOdel for Real Solvents (COSMO-RS),[53] heuristic computational models[54] and graph neural networks[55] have simplified the screening and selection of solvents for lignin solubilization through lignin-first biorefineries. These tools will enhance the efficiency of lignin-first biorefinery approaches. Overall, recent developments in this approach are expected to pave the way for efficient lignin fractionation and valorization. In recent years, lignin extraction using deep eutectic solvents has gained considerable attention because of its eco-friendly properties, mild operating conditions, good performance under atmospheric pressure, efficient lignin dissolution, reduced lignin degradation, reusability, and simple lignin recovery. However, the economic feasibility of using deep eutectic solvents for lignin separation on an industrial scale is still under investigation.

3.2. Opportunities and challenges in chemical depolymerisation of lignin

The production of aromatic compounds from lignin faces several major challenges including issues related to solubility, complexity, and heterogeneity. The depolymerisation of lignin produces a wide range of aromatic compounds, some of which can interfere with microbial growth and metabolism. Scaling up, feedstock variability, the need for cost-effective purification of aromatic compounds, and the energy-intensive nature of current processing methods further complicate lignin valorization. However, these challenges are not insurmountable. Reductive catalytic fractionation, oxidative depolymerisation, base-catalysed depolymerisation, acid-catalysed depolymerisation, electrocatalytic depolymerisation, enzymatic depolymerisation, and microbial depolymerisation are the main methods

used for lignin depolymerisation. Among the methods studied for lignin depolymerisation, reductive catalytic fractionation yields a higher monomer yield.[56] The extraction and purification of aromatic monomers following lignin depolymerization involve various techniques, including water extraction, distillation, use of organic solvents, ionic liquids, supercritical CO_2, membrane filtration, resins, and chromatography have been employed. A combination of these techniques is often used to obtain optimal extraction of aromatic compounds.[57] Sepabeads resin SP700 has been widely used for the purification of aromatic compounds because it offers a high surface area and porosity and is applicable to a wide range of pH values, regeneration efficiency, and scalability for industrial applications.[58,59] Most importantly, this process requires minimal use of solvents (methanol or ethanol) and is solvent-free, contributing to its cost-effectiveness and ecofriendly nature.

Researchers have successfully demonstrated the feasibility of integrating lignin extraction, depolymerization, purification of aromatic monomers, and production of valuable compounds in a closed-loop system. For example, Perez *et al.* developed a γ-valerolactone biorefinery approach for the isolation of lignin from poplar biomass. The depolymerization of lignin by hydrogenolysis using a palladium (Pd/C) catalyst and a liquid stream containing monomers was used to produce 2-pyrone-4,6-dicarboxylic acid (PDC) with a yield of 139 g kg^{-1} of lignin using *Novosphingobium aromaticivorans*. In addition, this approach has been successfully used to produce aromatic monomers from various biomass sources, including maple, sorghum, and switchgrass, demonstrating its adaptability to processing a wide biomass range.[60] Similarly, "plug-in processes of lignin" approach developed by integrating lignin and carbohydrate separation processes with lignin pretreatment methods, such as ammonia fiber expansion, dilute sulfuric acid pretreatment, steam explosion pretreatment, liquid-hot water pretreatment, and sodium hydroxide pretreatment. This integration effectively decreases the molecular weight of lignin, making it more suitable for conversion into PHA by *P. putida*.[61] These recent advancements demonstrate the possibility of developing effective and sustainable biorefinery processes that can utilize existing biorefinery instrumentation facilities to enhance the economic viability of lignin valorization. As research and development progress, these promising biorefinery approaches will be further optimized and will play a vital role in promoting a circular bioeconomy and reducing dependence on fossil resources.

3.3. Opportunities and challenges in electrocatalytic depolymerization of lignin

Electrocatalytic depolymerization of lignin is considered an eco-friendly approach because it provides relatively milder reaction conditions and electricity, making it attractive for lignin-based biorefinery applications.[62–64] This process utilizes homogeneous and heterogeneous catalysts and carbon, nickel, lead, platinum, and copper-based materials as electrodes for the oxidative or reductive cleavage of lignin.[64] Researchers have demonstrated the electrocatalytic degradation of aspen-derived lignin in a three-dimensional electrode reactor containing Pb/PbO_2 and stainless-steel wire as the anode and cathode, respectively. This process yielded 343.3 g of 4-methylanisole per kg lignin as the major component, along with vanillin, acetovanillone, syringaldehyde, 2,6-dimethoxy phenol, toluene, and styrene.[65] In another study, vanillin and syringaldehyde were produced from ethanol organosolv sweetgum lignin *via* electrooxidation in a three-electrode system containing a nickel foam electrode under ambient conditions.[66] Conversely, when the electrocatalytic degradation of kraft lignin was performed at elevated temperatures (160 °C), vanillin is selectively produced.[67] Notably, a recent study engineered a piolet-scale electrocatalytic system at the Technology Readiness Level (TRL) 6 for lignin depolymerization. This system uses an eco-friendly oxidizer, sodium peroxodicarbonate, to depolymerize kraft lignin through oxidative degradation, yielding 8 wt% of vanillin.[68]

Interestingly, Lindenbeck *et al.*, developed an eco-friendly electrocatalytic depolymerisation method utilising carbon as an electrocatalyst in a three-electrode system, which can depolymerise soda lignin into aromatic monomers. Subsequently, aromatic monomers undergo dearomatization to produce simple organic compounds, such as levulinate, 4-hydroxyvalerate, formate, and acetate.[64] These organic compounds can be utilized by bacteria as carbon sources to produce PHA. For example, engineered *Methylorubrum extorquens* AM1 overexpressing formate-tetrahydrofolate ligase (ftfL) has been reported to produce 11.07 g L^{-1} PHA using formate.[69] *Haloferax mediterranei*[70] and engineered *P. putida*[71] have been reported to produce PHA using levulinate. Additionally, engineered *E. coli*,[72] *P. putida*,[73] *Cobetia* sp. MC34, *Cobetia marina* DSM 4741T[74] and glycogen-accumulating microbes[75] can produce PHA using acetate. A recent study showed that *Halomonas* sp. strain JJY01 utilized acetate as a carbon source for PHA production.[76]

Electrocatalytic depolymerization of lignin offers a sustainable route for the production of aromatic monomers and organic compounds that are easily utilized by bacterial strains for PHA production. Additionally, this approach presents an eco-friendly operation, successful pilot-scale demonstrations, and easy manipulation of the reaction conditions. However, considering the production of a mixture of organic compounds by electrocatalytic depolymerization, genetic and metabolic engineering of microbes is necessary for their efficient utilization to produce PHA.

3.4. Opportunities and challenges in enzymatic depolymerization of lignin

In nature, microbes depend on enzymatic systems comprised of lignin depolymerization depends on lignin-modifying enzymes (LME) and lignin-degrading auxiliary enzymes (LDA). LME comprises laccases (Lac), manganese peroxidases (MnP), dye-decolorizing peroxidases (DyP), lignin peroxidases (LiP), versatile peroxidases (VP)and β-etherases.[77–81] LDA enzymes include aryl alcohol oxidases (AAO), cellobiose dehydrogenases

(CDH), glyoxal oxidases (GLOX), glucose oxidases, heme-thiolate haloperoxidases, pyranose 2-oxidases (POX), chloroperoxidases and aromatic peroxygenases.[77] LME primarily oxidizes lignin, leading to the cleavage of various linkages, including C–C and C–O–C, to produce aromatic monomers, dimers, trimers, and oligomers.[79,82] Metagenomic studies have shown the abundance of thioredoxin reductase (TRXB), glutathione peroxidase (GPX), and quinone reductases (nuoE and nuoF), indicating their involvement in lignin degradation.[83,84] LDA enzymes are not directly involved in lignin degradation but act in conjunction with LMEs by producing reactive oxygen species and are involved in redox reactions. This synergistic enzymatic reaction increased the overall yield of aromatic monomers from the lignin. In addition, the combination of the LME and LDA enzymes blocks the repolymerization of lignin. For example, the combination of β-etherase (LigE) from *Agrobacterium* sp. and Dyp or multicopper oxidase (CopA) from *P. putida* enhances lignin depolymerization and aromatic compound production.[85] Similarly, the combination of *Rhodococcus jostii* RHA1 glycolate oxidase and *Agrobacterium* sp., DyP peroxidase, produced various aromatic compounds from organosolv lignin.[86]

Incorporating LME and LDA into the fermentation process of PHA-producing bacteria, either through expression or addition, facilitates efficient utilisation of lignin as a carbon source. Studies have demonstrated that the addition of laccase to the fermentation medium enhances biomass production with a 17-fold increase in lipid yield in *Rhodococcus opacus* compared to laccase-free conditions.[87] Further improvements in growth and lipid production were observed when alkali lignin was depolymerized by *Trametes versicolor* laccase and the 1-hydroxybenzotriazole (HBT) system.[88] However, the molecular weight of lignin increased when treated with laccase and laccase HBT. To overcome lignin repolymerization, a combination of laccase or laccase + mediators + LDA enzymes can be used. For example, the addition of laccase + AAO + mediator in batch fermentation for lignin degradation resulted in the production of 3.34 g L^{-1} of bioplastic by *C. necator*, whereas without laccase + AAO + mediator, only 0.33 g L^{-1} bioplastic was produced.[89]

The addition of ionic liquids or deep eutectic solvents to the reaction medium facilitates the removal of lignin from biomass and enhances lignin depolymerization by enzymes.[90,91] For example, a bi-enzymatic system comprising 5% (v/v) of ionic liquid cholinium lysinate ([Ch][Lys]), lignin peroxidase, and aryl alcohol oxidase reduced the molecular weight of lignin and produced phenol and 1,2-dimetohxybenzene.[92] Similarly, lactic acid: betaine-based deep eutectic solvents have been shown to enhance the activity of *Myceliophthora thermophila* laccase by up to 300%.[93] Considering the cost of purified enzymes, overexpression or heterologous expression of ligninolytic enzymes in target bacteria is viewed as an economical and promising strategy. Recent studies have demonstrated the successful application of this approach in major PHA-producing strains. For instance, heterologous expression and extracellular secretion of Streptomyces coelicolor A3(2) laccase in *P. putida* A514, by harnessing the power of the type I secretion system, promoted growth and lignin depolymerization.[94] Similarly, the engineered type I secretion system enhanced laccase secretion, enabling *Halomonas* sp. Y3 to produce 1.31 g L^{-1} PHA from lignin.[95] Researchers have further explored the possibility of cell-surface interplay of lignin-degrading enzymes for the production of aromatic compounds for valorization. A recent study showed that *P. putida* KT2440, displaying an engineered laccase CotA (T260/L385K/F416R) from *Bacillus coagulans* on its cell surface, can grow well on lignin-containing medium when compared to its wild-type counterpart.[96] Similarly, a recent study by Liang *et al.* demonstrated the enhanced depolymerisation and reduction of the molecular weight of lignin by *P. putida* A514 upon supplementation with a synthetic enzyme cocktail comprising 15 enzymes.[97] These studies suggest that the low efficiency of lignin degradation by bacteria and repolymerisation which restricts the production of aromatic compounds, can be overcome by the overexpression of LME and LDA or by directly incorporating them into the fermentation medium.

3.5. Opportunities and challenges in bioelectrochemical depolymerization of lignin

Recent advancements, such as the development of bioelectrochemical methods, offer an eco-friendly alternative by eliminating the requirement of harsh reaction conditions used for the chemical, physical, and physicochemical depolymerization of lignin. For example, Mishyn *et al.* engineered a membraneless β-etherolytic bioanode-based bio-electrocatalytic system with NAD regenerating potential containing immobilised NAD-dependent Cα-dehydrogenases (LigD and LigL), glutathione-dependent etherases (LigE and LigF), and a glutathione-dependent lyase (LigG), which are derived from *Sphingobium paucimobilis* SYK-6 and can cleave the β-O-4 linkage of lignin.[98] Similarly, a Ligninolytic Hybrid Air-Breathing Biocathode comprising gas diffusion electrode/oxidized carbon nanotubes/5,10,15,20-tetrakis(4-methoxyphenyl)-21*H*,23*H*-porphine cobalt (II)/pyrene-modified linear poly(ethyleneamine)/*Phanerochaete chrysosporium* LiP (GDE/OCNT/CoTMPP/Py-LPEI/LiP) can depolymerize lignin at room temperature using oxygen from air. Despite these advantages, this biocathode retains only 50% of its activity for up to three cycles.[99] Therefore, further improvements in the reusability of the electrode are necessary for large-scale applications to ensure the efficient and economical depolymerization of lignin.

Studies have shown that microbial peroxide-producing cells can be used to depolymerize kraft lignin through H_2O_2 mediated oxidation in batch mode and fed-batch mode with self-life of 10 and 21 days, respectively. Further downstream analysis revealed the presence of ferulic acid, vanillin, 4-butoxybenzaldehyde, guaiacol, phenylacetaldehyde, homovanillyl alcohol and other aromatic compounds.[100] The versatility of this technique has been demonstrated by its application in depolymerizing rice straw lignin, indicating its compatibility with depolymerizing lignin from diverse biomasses.[101] In a

recent study, a combination of H_2O_2 produced in microbial electrochemical cells and a methyltrioxorhenium catalyst selectively depolymerized kraft lignin through oxidation and produced guaiacol as a major aromatic compound.[102] These techniques utilize a two-chamber system: an anode chamber containing domestic wastewater enriched with nutrients as an anolyte to promote microbial growth and biofilm formation, and a cathode chamber holding lignin-containing solution as a catholyte. Future studies focusing on the application of bioelectrochemical methods for lignin depolymerization should address various key issues such as product selectivity, scalability, catalyst optimization, environmental and sustainability aspects, and economic feasibility to revolutionize the eco-friendly production of aromatic compounds from lignin.

4. Recent trends and opportunities in engineering of microorganisms for PHA production from lignin

PHA is a reserve carbon source produced by microorganisms in response to environmental stresses, including nitrogen, phosphate or oxygen limitation and excess carbon supply.[103] The conversion of lignin to valuable products is achieved primarily through metabolic pathways in microbes, which are often referred to as "biological funnelling". These pathways are divided into three: upper pathway, funnelling pathway and lower pathway.[19] The upper pathway involves microorganisms obtaining LDACs or lignin monomers, including ferulic acid, *p*-coumaric acid, and vanillic acid, from the environment. These are then converted to key intermediates, such as PCA and catechol, through various upper pathways present in microorganisms. The These key intermediates enter central carbon metabolism *via* aromatic ring cleavage pathways.[104] Briefly, PCA and catechol undergo ring opening to form, acetyl-CoA and succinyl-CoA,[105] which subsequently enter central carbon metabolic pathways such as TCA and fatty acid synthesis pathways[105,106] to produce PHAs and triglycerides. The specific products synthesized depend on the performance of the microorganisms involved.[107]

The production of PHA from lignin and LDACs remains an emerging area of research, primarily explored using members of the *Pseudomonas*,[108] *Halomonas*, and *Cupriavidus* genera. While lignin shows potential as a renewable carbon source, alternatives such as sugars, fatty acids, lignocellulosic hydrolysates, and food waste currently yield higher PHA concentrations[109] (Tables 1 and 2). For instance, *C. necator* H16 can produce >100 g L^{-1} of PHA using waste cooking oil and sucrose as carbon sources.[110] In the case of lignin- or lignin-rich alkaline pretreated liquor as a carbon source, PHA production of ~18 mg L^{-1}–4.5 g L^{-1} PHA production were reported (Table 1). Notably, *Halomonas alkalicola* M2 achieved a maximum output of 1.89 g L^{-1} PHA using laccase-pretreated alkali lignin,[111] whereas *C. necator* DSM 545 reached 4.47 g L^{-1} PHA with alkaline-pretreated corn stover liquor supplemented with laccase, AAO, mediators, silica nanoparticles Aerosol R816, and solubility-enhancing agents.[89] Similarly, *P. putida* produced ~4.5 g L^{-1} PHA from a soluble lignin stream from steam-explosion-pretreated corn stover.[61] Although the yield of PHA from lignin and LDACs is lower than that from other carbon sources, it is comparable when considering the grams of PHA produced per gram of substrate. Nevertheless, the overall PHA yield from lignin-based substrates is lower. This indicates that although lignin and its derivatives can produce PHA at a rate similar to the amount of substrate consumed, they may not match the total PHA production efficiency of alternative carbon sources. This variation highlights the need for further optimisation of lignin utilisation to enhance its viability as a feedstock for PHA production. The primary reason for this discrepancy is the inherent toxicity and inefficient metabolic pathways of lignin and LDACs, which often hinder bacterial growth.

P. putida KT2440 is a versatile bacterium widely studied for its ability to metabolize lignin and LDACs as carbon source to produce PHA.[108] Its exceptional adaptability to diverse environmental conditions, non-pathogenic nature, ease of genetic modification, and ability to utilize a wide range of carbon sources makes it a microbial cell factory for PHA synthesis at an industrial level.[112] Several studies have demonstrated that *P. putida* KT2440 can synthesis PHA from alkaline pretreated liquors, lignin containing steams and various aromatic compounds (Tables 1 and 2). PHA is present within the cells as a reserve carbon source. Consequently, following the cessation of environmental stress, PHA is degraded by the depolymerizing enzyme PhaZ. In addition, lignin depolymerization and degradation of aromatic compounds by native pathways are not efficient in producing more PHA. To address these limitations, researchers have engineered bacteria by optimizing the upper pathways supplying metabolic intermediates through knockout, overexpression, or heterologous expression of appropriate genes (Fig. 4). Genetic and metabolic engineering mainly focuses on (i). blocking (*R*)-3-hydroxyacyl-CoA through the β-oxidation pathway to increase the availability of PHA precursors (ii). overexpression of genes involved in PHA synthesis by introducing different ribosome-binding sites (RBS) and/or under strong promoters *via* chromosomal integration or expression vectors (iii). enhancement of reducing equivalents such as NADH or NADPH supply to enrich carbon flux for PHA synthesis (iv). overexpression of aromatic compound uptake transporter proteins (v). attenuation of PHA depolymerization (vi). genome reduction (vi). cell morphology engineering (vii). adaptive laboratory evolution (Fig. 4).

4.1. Genetic engineering

For the first time, Linger *et al.*, demonstrated the capability of *P. putida* KT2440 to produce mcl-PHA through its intrinsic "biological funnelling pathways". These pathways enable the transformation of a mixture of aromatic compounds present in alkaline pretreated liquor (APL) into central intermediates (catechol and PCA) under nitrogen-limited conditions. This is followed by the entry of central intermediates into the central carbon metabolism through aromatic ring cleavage pathways

Table 1 List of bacteria producing PHA from lignin and lignin rich alkaline pretreated liquor from different biomasses

Strains and genetic modifications	Substrate and fermentation conditions	PHA concentration (mg L^{-1})	PHA yield (mg g^{-1})	Ref.
P. putida KTYY06 Δ*PhaZ*-Δ*aldB*::*PhaC*-Δ*FadBA* + overexpressing of *PhaC*, *PhaJ4*, and *PhaG*	Alkaline pretreated liquor (30 g L^{-1}), octanoic acid (1 g L^{-1}). Fed-batch, flask, M9 medium, 30 °C, 180 rpm, 72 h.	~930	~31	153
P. putida KT2440 wild-type	M9 medium, pH = 7.2, euphorbia C-lignin (3 g L^{-1}), 30 °C, 180 rpm, 72 h, batch mode in flasks.	~137	~45.66	229
Pseudomonas monteilii BCC19149 wild-type	Minimal medium supplemented with lignin rich stream derived from sugarcane bagasse, pH = 7.0, 30 °C for 120 h, pulse-feeding, bioreactor.	~238	—	178
Genome reduced *P. putida* KTU-U13	M9 minimal medium containing alkaline pretreated liquor (~20 g of lignin per L), pH = 7.0, 30 °C, 180 rpm, for 48 h, fed-batch mode in flasks.	~1400	~70	180
P. putida H Δ*catA2*	M9 minimal medium containing lignin stream enriched with catechol (250 mM) pH = 7.0, 30 °C, for 48 h, batch mode in bioreactor.	~1400	—	230
Pseudomonas sp. Hu109A wild-type	Mineral salt medium supplemented with alkali lignin (3 g L^{-1}), pH = 7.0, 37 °C, 220 rpm, 220 rpm, 54 h, batch mode in flasks.	~186	~62	231
P. putida NX-1 wild-type	M9 medium supplemented with kraft lignin (10 g L^{-1}), pH = 7.0, 30 °C, 200 rpm for 7 days, batch mode in flasks.	~114	~11.4	232
P. putida KT2440 (C1J4) overexpressing of *phaC1* and *phaJ4*	M9 medium supplemented with 20 g L^{-1} of soluble lignin stream: enzymatic hydrolysate (75 : 25), pH = 7.0, 28 °C, and 200 rpm for 20 h, fed-batch mode in flasks.	~1380	~69	181
P. putida KT2440 Engineered strain	Soluble lignin stream (pH = 7.0) from ammonia fiber expansion of corn stover supplemented with mineral salts, 28 °C, 200 rpm for 24 h, fed-batch mode in flasks.	~3600	—	61
	Soluble lignin stream (pH = 7.0) from dilute sulfuric acid pretreated corn stover supplemented with mineral salts, 28 °C, 200 rpm for 24 h, fed-batch mode in flasks.	~2200	—	61
	Soluble lignin stream (pH = 7.0) from steam explosion pretreated corn stover supplemented with mineral salts, 28 °C, 200 rpm for 24 h, fed-batch mode in flasks.	~4500	—	61
	Soluble lignin stream (pH = 7.0) from liquid hot water pretreated corn stover supplemented with mineral salts, 28 °C, 200 rpm for 24 h, fed-batch mode in flasks.	~3200	—	61
	Soluble lignin stream (pH = 7.0) from sodium hydroxide pretreated corn stover supplemented with mineral salts, 28 °C, 200 rpm for 24 h, fed-batch mode in flasks.	~2300	—	61
P. putida AG2162 Δ*phaZ* Δ*fadBA1* Δ*fadBAE2* ΔaldB::Ptac-*phaG-alkK-phaC1-phaC2*	Lignin-containing stream derived from corn stover supplemented with M9 mineral salts, 30 °C, 225 rpm for 78 h, batch mode in flasks.	~116	—	113
P. putida KT2440 wild-type	Corn stover derived soluble lignin stream rich in ferulic acid and *p*-coumaric acid, residual sugar, supplemented with mineral salts, pH = 7.0, 28 °C, and 200 rpm for 18 h, fed-batch mode in flasks.	~1500	—	182
P. putida KT2440	Alkaline pretreatment liquid (pH = 7.0) from corn stover supplemented with M9 salts, 30 °C, 225 rpm for 48 h, batch mode in bioreactor.	~252	—	105
Pandoraea sp. ISTKB wild-type	Mineral medium containing kraft lignin (2 g L^{-1}) 30 °C, 185 rpm for 4 days, batch mode in flasks.	~18	~9	176
Halomonas alkalicola M2 wild-type	M9 medium (pH = 10.0) containing NaCl 70 g L^{-1}, 10 g L^{-1} Sigma alkali lignin pretreated with laccase + ABTS for 24 h, and the seeded with *H. alkalicola* M2, 30 °C, 200 rpm for 7 days, non-sterilized fermentation, batch mode in flasks.	~1890	~189	111
Halomonas sp. Y3_05[a] *Halomonas* sp. Y3_05[b]	Co-cultivation of Y3_05 and Y3_08 in modified M9 medium (pH = 9.0) containing NaCl 60 g L^{-1}, alkaline lignin 10 g L^{-1}, 1 mM ABTS, 30 °C, 200 rpm for 7 days, non-sterilized fermentation, batch mode in flasks.	~740	~74	95
	Co-cultivation of Y3_05 and Y3_08 in alkaline pretreated liquor (100%) derived from bamboo supplemented with M9 salts and NaCl 60 g L^{-1} (pH = 9.0), 30 °C, 200 rpm for 7 days, non-sterilized fermentation, batch mode in flasks.	~1314	—	95
Halomonas sp. Y3_08[a] *Halomonas* sp. Y3_08[b]	Co-cultivation of Y3_05 and Y3_08 in alkaline pretreated liquor (100%) derived from bamboo supplemented with M9 salts and NaCl 60 g L^{-1} (pH = 9.0), 30 °C, 200 rpm for 7 days, batch mode in flasks.	~1208	—	95
Halomonas sp. Y3-coABTS Δ*PrpC*-Δ*PcaG*-Δ*PcaH*-Δ*PhaZ*, heterologously expressing laccase	Modified M9 medium (pH = 9.0) containing NaCl 60 g L^{-1}, lignin 6 g L^{-1}, ABTS, 30 °C, 200 rpm for 7 days.	~425.82	~70.97	156

Table 1 *(Contd.)*

Strains and genetic modifications	Substrate and fermentation conditions	PHA concentration (mg L^{-1})	PHA yield (mg g^{-1})	Ref.
Burkholderia epacian B1-2 wild-type	Alkaline pretreated liquor (pH was adjusted to 7.0) containing mineral salts, nitrogen source 70 mg L^{-1}, batch mode in flasks.	~87.2	—	151
Cupriavidus necator DSM 545	Mineral salt medium containing with alkaline pretreatment liquor from corn stover and supplements (laccase, AAO, mediators, silica nanoparticles Aerosol R816, Tween 80 and dimethyl sulfoxide (DMSO)) pH = 6.8, 32.5 °C, 400 rpm, 56 h, fed-batch (supplements) and pulse feeding (alkaline pretreatment liquor), fermenter.	~4470	—	89
C. necator DSM 545	Mineral salt medium containing with alkaline pretreatment liquor from corn stover and supplements (laccase, AAO, mediators, silica nanoparticles Aerosol R816, Tween 80 and DMSO) 32.5 °C, 250 rpm, 7 days, fed-batch mode in flasks.	~2100	—	233
Cupriavidus basilensis B-8 wild-type	Lignin rich alkaline pretreated liquor of rice straw (pH was adjusted to 7.0) containing mineral salts, 30 °C, 150 rpm, batch mode in flasks.	~482.7	—	179
C. basilensis B-8 wild-type	Mineral salt medium containing 5 g L^{-1} of kraft lignin, nitrogen source 65 mg L^{-1}, 30° C, 150 rpm, 7 days, fed-batch mode in flasks.	~319.4	~63.8	177
C. basilensis B-8 wild-type	Mineral salt medium containing 5 g L^{-1} of kraft lignin, nitrogen source 65 mg L^{-1}, 30° C, 150 rpm, 7 days, batch mode in flasks.	~128	~25.6	177

[a] Over expressing laccase. [b] Δ*PhaZ*, overexpressing *phaA*, *phaB* and *phaC*.

to produce PHA.[105] Subsequently, many researchers have focused on engineering *P. putida* KT2440 to improve PHA production. For example, knockout of *PhaZ* is involved in depolymerization of PHA, and *FadB* (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase) and *FadA* (3-ketoacyl-CoA thiolase) are involved in β-oxidation of fatty acids. Additionally, overexpression of genes involved in (*R*)-3-hydroxyacyl-CoA such as *phaG* (hydroxyacyl-ACP acyl-transferase), *AlkK* (acyl-CoA-synthase), and PhaC1 and PhaC2 are involved in polymerization. These genetic modifications resulted in significantly enhanced PHA production in the engineered strain using *p*-coumaric acid and a lignin-containing stream when compared to the wild-type.[113] It has been shown that aromatic ring-cleavage pathways can be swapped through genetic engineering to synthesize a target product. For example, substitution of the *ortho*-degradation pathway of catechol in *P. putida* KT2440 with the 4,5-*meta* cleavage pathway from *P. putida* mt-2 enhanced pyruvate yield. Similarly, swapping of the *ortho*-cleavage pathway with the heterologous *meta*-cleavage pathway of *Sphingobium* sp. SYK-6 in *P. putida* KT2440 enhanced pyruvate production 5-fold using *p*-coumaric acid as a substrate.[114] When phaP1 from *Cupriavidus necator* H16 was expressed in *Rhodopseudomonas palustris* CGA009, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) was produced using *p*-coumaric acid as a substrate, whereas wild-type *R. palustris* failed to produce PHBV.[115]

4.2. Promoter engineering

Promoter engineering has the potential to regulate transcription and stabilize the expression of specific genes. Exploring the possibility of combining promoter engineering with other genetic modifications, such as the optimization of metabolic pathways and substrate utilization, may improve PHA production. *P. putida* MA-6 equipped additional copies of catechol 1,2-dioxygenases (catA and catA2) under the control of P_{cat} promoter through chromosomal integration has been reported to produce 64.2 g L^{-1} of muconic acid from catechol.[116] Similarly, chromosomal integration of phenol hydrolase genes (dmpKLMNOP) derived from *P. putida* CF600 under the control of the PGRO promoter into *P. putida* MA-6 enabled the production of muconic acid (12 g L^{-1}) using hydrothermally depolymerized pine lignin.[116] Zhao *et al.*, have identified several strong endogenous promoters in *Pseudomonas mendocina* NK-01 through transcriptomic analysis and neural network promoter prediction. Further studies involving integration of selected promoters into the chromosomal region upstream of phaC have shown enhanced PHA production.[117] Insertion of a constitutive promoter P_{porin} in the upstream of the PHA synthesis-promoting gene *PhaCAB* has been demonstrated to enhance PHA production from wheat straw liquor by *Halomonas elongata* A1.[118] These successful genetic modifications highlight the prospects for further improving the potential of *P. putida* to biotransform LDACs into commercially important products. Subsequent studies should focus on optimizing the expression levels of key enzymes or exploring additional metabolic pathways to improve the central metabolite levels, which will facilitate the development of more efficient and multifaceted *P. putida* strains suitable for lignin biorefineries.

In addition to endogenous promoters, plasmid-based expression of genes in *P. putida* KT2440 inducible promoters such as P_m (3-methylbenzoate inducible), P_{rhaB} (rhamnose inducible), P_{saI} (salicylate inducible), and P_{araB} (arabinose inducible) have been reported.[119] Advancements in machine

Table 2 List of bacteria producing PHA from lignin-derived aromatic compounds and other carbon sources

Strains and genetic modifications	Lignin derived aromatic compounds and fermentation conditions	PHA concentration (mg L^{-1})	PHA yield (mg g^{-1})	Ref.
P. putida KTYY01 Δ*PhaZ*	M9 medium, pH = 7.0, ferulic acid (4 g L^{-1}) and octanoic acid (1 g L^{-1}) 30 °C and 180 rpm, for 24 h, batch mode in flasks.	~1320	~330	153
P. putida KTYY04 Δ*PhaZ*-Δ*aldB*:: *PhaC* + overexpressing of *PhaC*, *PhaJ4*, and *PhaG*	M9 medium, pH = 7.0, *p*-coumaric acid (4 g L^{-1}) and octanoic acid (1 g L^{-1}), 30 °C and 180 rpm, for 72 h, batch mode in flasks.	~1140	~285	
P. putida KTYY06 Δ*PhaZ*-Δ*aldB*:: *PhaC*-Δ*FadBA* + overexpressing of *PhaC*, *PhaJ4*, and *PhaG*	M9 medium, pH = 7.0, *p*-coumaric acid (4 g L^{-1}) and heptanoic acid (1 g L^{-1}), 30 °Cand 180 rpm, for 24 h, batch mode in flasks.	~1180	~295	
	M9 medium, pH = 7.0, *p*-coumaric acid (4 g L^{-1}) and octanoic acid (1 g L^{-1}), 30 °C and 180 rpm, for 72 h, batch mode in flasks.	~1230	~307	
	M9 medium, pH = 7.0, ferulic acid (4 g L^{-1}) and heptanoic acid (1 g L^{-1}) 30 °C and 180 rpm, for 72 h, batch mode in flasks.	~1360	~340	
	M9 medium, pH = 7.0, ferulic acid (4 g L^{-1}) and octanoic acid (1 g L^{-1}) 30 °C and 180 rpm, for 72 h, batch mode in flasks.	~1150	~287	
	M9 medium, *p*-coumaric acid (12 g L^{-1}) and octanoic acid (2 g L^{-1}) 30 °C, 180 rpm, 36 h, fed-batch mode in flasks.	~2460	~205	
P. putida H Δ*catA2*	M9 medium, pH = 6.9 ± 0.1, 30 °C, 300–1200 rpm to maintain 20% saturation of dissolved oxygen level, oxygen flow 3 l min^{-1} (air : oxygen = 5 : 1), batch culturing on 10 mM benzoate for 12 h, followed by exponential feeding up to 40 h and constant feeding (8.3 g h^{-1}) up to 80 h.	~6100	—	230
P. putida KT2440	Mineral salt medium supplemented with a mixture of *p*-coumarate, ferulate, and benzoate (5 g L^{-1}), 30 °C, pH = 7.0, 300 rpm, 72 h, batch mode in bioreactor.	~582	~116.4	171
P. putida K-pAhprada Δpca-pAhrpada (*hps*, *phi*, and *ada*)	M9 medium, pH = 7.2, 30 °C, ethanol assisted depolymerized lignin, batch mode in flasks.	~303	—	234
P. putida AG2162 Δ*phaZ* Δ*fadBA1* Δ*fadBAE2* ΔaldB::Ptac-*phaG-alkK-phaC1-phaC2*	M9 minimal medium, *p*-coumaric acid (13 g L^{-1}), 30 °C, 225 rpm for 85 h, high-cell density, feed batch mode in flasks.	~953	~73.30	113
P. putida strain A514 Overexpressing *phaG*, *alkK*, and *phaC1*	M9 medium containing 2.5 g L^{-1} of vanillic acid, 30 °C, 200 rpm for 50 h, batch mode in flasks.	~246	~98.4	235
Pandoraea sp. ISTKB wild-type	Mineral medium (pH = 8.0) containing 4-hydroxybenzoic acid (3 g L^{-1}), 30 °C, 185 rpm for 4 days, batch mode in flasks.	~246	~82	176
Halomonas sp. Y3-16 Δ*PrpC*-Δ*PcaG*-Δ*PcaH*-Δ*PhaZ*, overexpressing *DesA* and *LigM*	Modified M9 medium (pH = 9.0) containing NaCl 60 g L^{-1}, 4-hydroxybenzoic acid 1 g L^{-1}, 30 °C, 200 rpm for 48 h, batch mode in flasks.	~716	~716	156
	Modified M9 medium (pH = 9.0) containing NaCl 60 g L^{-1}, syringic acid 1 g L^{-1}, 30 °C, 200 rpm for 48 h, batch mode in flasks.	~449	~449	156
	Modified M9 medium (pH = 9.0) containing NaCl 60 g L^{-1}, vanillic acid 1 g L^{-1}, 30 °C, 200 rpm for 48 h, batch mode in flasks.	~488	~488	156
Halomonas sp. Y3-18 Δ*PrpC*-Δ*PcaG*-Δ*PcaH*-Δ*PhaZ*, overexpressing *DesA*, *LigM* and *PobA*	Modified M9 medium (pH = 9.0) containing NaCl 60 g L^{-1}, each 0.5 g L^{-1} vanillic acid, 4-hydroxybenzoic acid, and syringic acid, 30 °C, 200 rpm for 48 h, batch mode in flasks.	~522	~348	156
Halomonas sp. Y3 wild-type	Modified M9 medium (pH = 9.0) containing NaCl 60 g L^{-1}, 4-hydroxybenzoic acid 2 g L^{-1}, 30 °C, 200 rpm, 72 h, batch mode in flasks.	~535.2	~267.6	152
	Modified M9 medium (pH = 9.0) containing NaCl 60 g L^{-1}, protocatechuate 2 g L^{-1}, 30 °C, 200 rpm, 72 h, batch mode in flasks.	~506.5	~253.2	
	Modified M9 medium (pH = 9.0) containing NaCl 60 g L^{-1}, catechol 2 g L^{-1}, 30 °C, 200 rpm, 72 h, batch mode in flasks.	~435.6	~217.8	
	Modified M9 medium (pH = 9.0) containing NaCl 60 g L^{-1}, vanillic acid 2 g L^{-1}, 30 °C, 200 rpm, 72 h, batch mode in flasks.	~440.8	~220.8	
Burkholderia cepacia B1-2 wild-type	Mineral salt medium (pH = 7.0) containing 3 g L^{-1} of *p*-hydroxybenzoic acid, nitrogen source 70 mg L^{-1}, 30 °C, 180 rpm, 72 h, fed-batch mode in flasks.	~1420	~473	151
Burkholderia sp. ISTR5 (R5) wild-type	Mineral salt medium, *p*-Coumaric acid + molasses, 30 °C, 165 rpm, 7 days, batch mode in flasks,	~270	—	48
Ralstonia eutropha strain H16 wild-type	Mineral salt medium containing 10 g L^{-1} of 3-hydroxybenzoic acid, 72 h, 30 °C, 130 rpm, batch mode in flasks.	~1600	~160	236
	Mineral salt medium containing 10 g L^{-1} of 4-hydroxybenzoic acid, 72 h, 30 °C, 130 rpm, batch mode in flasks.	~690	~69	

Strains and genetic modifications	Sugars and other feedstocks and fermentation conditions	PHA concentration	PHA yield	Ref.
Azotobacter vinelandii	Mineral salt medium supplemented with 87.8 g L^{-1} of glucose, pH = 7.0, 30 °C at 150 rpm, 28 h, fed-batch mode in a fermenter.	~30.3 g L^{-1}	~345	237

Table 2 *(Contd.)*

Strains and genetic modifications	Sugars and other feedstocks and fermentation conditions	PHA concentration	PHA yield	Ref.
Cupriavidus necator H16	Mineral salt medium supplemented with 150 g L^{-1} of waste cooking oil, pH = 7.0, 30 °C, 180 rpm, 66 h, fed-batch mode in flasks.	~114.1 g L^{-1}	~760	110
	Mineral salt medium supplemented with 90 g L^{-1} of waste fish oil, pH = 7.0, 30 °C, 180 rpm, 48 h, fed-batch mode in flasks.	~83.2 g L^{-1}	~920	110
Cupriavidus necator H16	Mineral salt medium supplemented with oleic acid 15 g L^{-1}, pH = 7.0, 30 °C, 150 rpm 144 h, batch mode in flasks.	~6.715 g L^{-1}	~447	238
Cupriavidus necator TISTR 1335	Hydrogenic effluent (33.51 g COD per L) from biohythane pilot plant, pH = 6.87, 30 °C, 150 rpm, non-sterile fermentation, batch mode in flasks.	~3.02 g L^{-1}	—	239
Halogeometricum borinquense strain E3	Norberg and Hofstein medium containing acid hydrolysed cassava waste (10% v/v), pH = 7.0, 37 °C, 110 rpm, 10 days.	~1.52 g g L^{-1}	—	240
Salinicola salarius ES021	Modified mineral medium containing sugarcane molasses (5%), NaCl (3%), 30 °C, pH ~ 7.0, aeration rate = 2.5 vvm, 150 rpm, 48 h.	~12.88 g L^{-1}	—	240
Cupriavidus necator H16 expressing sucrose hydrolase (*cscA*) and sucrose permease (*cscB*)	Mineral salt medium containing sucrose as carbon source, pH = 6.7–6.8, 30 °C, 65 h, 500 rpm high-cell-density fermentation, fed-batch mode in a bioreactor.	~113 g L^{-1}	~400	241
Ralstonia eutropha ATCC 17699	Mineral salt medium containing 30 g L^{-1} of Na_2CO_3 + Na_2SO_3 pretreated *Hibiscus cannabinus* L. biomass enzymatic hydrolysate, pH = 7.0, 30 °C, 200 rpm, 36 h.	~10.10 g L^{-1}	~488	242
Paracoccus sp. LL1	Modified mineral salt medium (10 g L^{-1} NaCl) supplemented enzymatic hydrolysate of corn stover (total sugar concentration = 40 g L^{-1}), 30 °C, pH = 7.5, aeration rate = 2 vvm, 700 to 1300 rpm to maintain 20% saturation of dissolved oxygen level, 72 h, batch mode in fermenter.	~9.71 g L^{-1}	~251	243
Engineered *P. putida EM42*	Mineral salt medium supplemented with crude glycerol (20 g L^{-1}), 30 °C, pH = 7.0, agitation was adjusted throughout the fermentation to maintain 40% saturation of dissolved oxygen level, 180 h, fed-batch mode in fermenter.	~49.5 g L^{-1}	—	244
Ralstonia eutropha	Mineral salt medium supplemented with carob pulp extract (sugar content 40 g L^{-1}) 28 °C, pH = 7.0, aeration rate = 0.8 vvm, 250 rpm, 60 h, batch mode in fermenter.	~12.2 g L^{-1}	~0.305	245

learning and artificial intelligence have significantly boosted the development of highly efficient promoter prediction tools. For example, machine learning and duplex stability-based promoter prediction in prokaryotes (MLDSPP)[120] have utilized these technologies to improve the precision in predicting promoters from genome sequences. Similarly, artificial intelligence and machine learning have been instrumental in developing σ70-based synthetic promoter libraries,[121] designing *de novo* synthetic promoters,[122] and predicting promoter strength from constitutive (P_{trc}) promoter libraries.[123] These innovations are expected to pave the way for magnifying the expression of the genes/pathways necessary for the utilization of LDACs in industrially important bacterial strains.

4.3. Transporter proteins

Microbes utilize porin, TonB-dependent ATP-binding cassette, tripartite ATP-independent periplasmic, and major facilitator superfamily transporters for the uptake of aromatic compounds.[124] These transporter systems vary in selectivity and efficacy, with certain transporters exhibiting high specificity for particular aromatic compounds, whereas others possess wider substrate specificities. Thus, the selection of transporter systems for overexpression or heterologous expression in engineered microbial strains based on LDACs to be transformed is of paramount importance. Wada *et al.* (2021)[125] identified and characterized aromatic acid/H + symporters, such as PcaK, HcnK, and VanK, in *P. putida* KT2440, which are involved in the uptake of LDACs. In brief, PcaK facilitates the uptake of PCA and 4-hydroxybenzoate, HcnK is involved in the uptake of ferulic acid and *p*-coumaric acid, and VanK mediates the uptake of vanillic acid and PCA.[125] Subsequently, co-expression of the transporter protein HcnK, vanillate-*O*-demethylase (VanAB), and 4-hydroxybenzoate hydroxylase (PobA) under the control of an autoregulatory system containing a biosensor resulted in the production of 12.7 g L^{-1} of PCA using ferulic acid and *p*-coumaric acid.[126] In a recent study, utilizing transporter deletion libraries, researchers identified various transport proteins in *Escherichia coli* and *Saccharomyces cerevisiae* that are responsible for transporting aromatic compounds, including vanillin.[127] These findings highlight the potential of overexpressing or heterologously expressing these transporter proteins in industrially relevant bacterial strains to enhance the efficiency of production of valuable compounds from lignin.

4.4. Genome reduction

Genome reduction or streamlining focuses on the deletion of genomic islands (GIs) acquired by microbes through horizon-

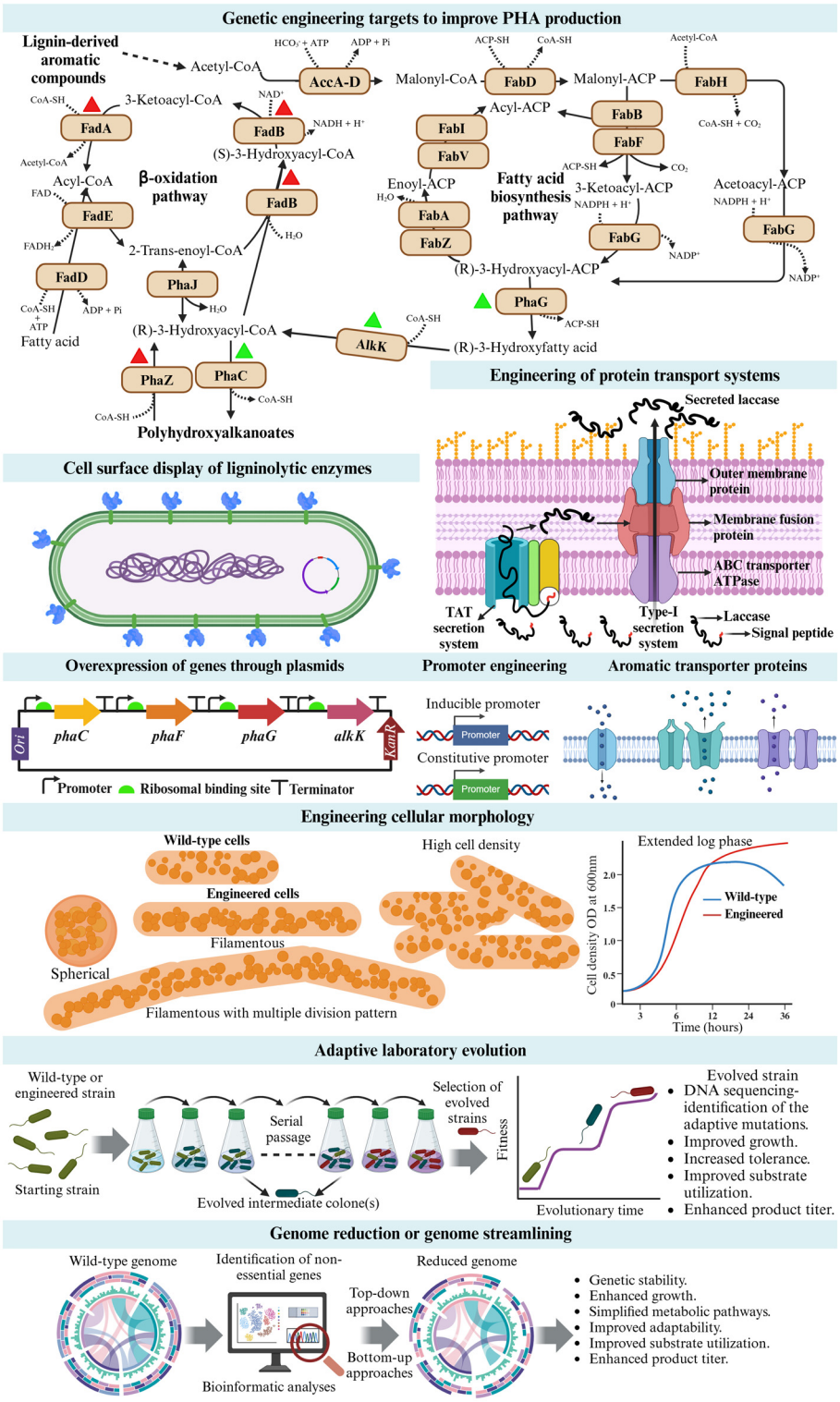

Fig. 4 Strategies to enhance PHA production in bacteria from lignin-derived aromatic compounds. Created using BioRender (https://BioRender.com).

tal gene transfer, which are not essential for their survival or metabolism. It offers genetic stability, enhanced growth, simplified metabolic pathways,[128] improved adaptation to environmental conditions,[129] enhanced substrate utilization, energy and redox balance and product yield. Advanced and high-throughput computational technologies, such as comparative genome analyses, metabolic modelling, and genome-scale models, enable the prediction of minimal genes required for

growth, biomass production, and target product synthesis. Integration of experimental data into computational predictions allows refining and accuracy for genome reduction in industrially important strains. Homologous recombination and CRISPR-mediated approaches are widely used to achieve genome streamlining.[130] For example, the genome-reduced *P. putida* EM383 strain, which had 4.3% of the reduced genome of its parental strain *P. putida* KT-2440, showed better tolerance to oxidative stress, stationary phase survival, biomass production, and expression of heterologous genes.[131] Similarly, genome reduced *P. putida* KTU-U27Δ*gcd* expressing *phaC1*, *phaC2* and pyruvate dehydrogenase (*acoA*) under the control of endogenous promoter P46, achieves 3.01 g L^{-1} of PHA using glucose as a substrate.[132] Likewise, genome-reduced *P. putida* KTU-U13 exhibited better degradation of γ-hexachlorocyclohexane and 1,2,3-trichloropropane through the integration of degradation pathways into its chromosome. In addition, compared to the wild-type strain, the genome-reduced strain harbouring the plasmid with the zeaxanthin biosynthesis pathway showed stable expression and zeaxanthin production.[133] These findings underscore the importance of genome reduction for improving PHA production. However, the impact of genome reduction on PHA production from lignin and LDACs remains to be evaluated.

4.5. Engineering cellular morphology

The morphology and growth behavior of the cell influence the accumulation of intracellular products, and thus modification through bacterial morphological engineering not only enhances yield but also facilitates downstream isolation.[134] Reducing the amount of peptidoglycan in the cell wall may allow the cell membrane to soften and expand more easily to store more products.[134] Overexpression of a repressor gene (SulA), which manipulates cell division, has been observed to block normal binary cell division. This results in filamentation of the cell, growth of cell size, and expansion of the space for storing PHA.[135] Concomitantly, filamentation enables cells to intertwine and settle under the influence of gravity, thereby facilitating the subsequent separation of cells from the culture medium.[136] Changing rod cell morphology to spherical cell morphology is another way to reduce cell space limitations.[137] Modulation of cellular morphology and growth kinetics in *Escherichia coli* JM109[138] and *Pseudomonas mendocina* NK-01[139] has been documented to enhance PHA production from glucose. Engineered strains showed alterations in cellular morphology, characterized by filamentous cells, and were accompanied by an extended log phase, facilitating the production of more PHA over time compared to the wild-type strains.

4.6. Adaptive laboratory evolution (ALE)

ALE is a widely used and simple method to enhance various characteristics of industrially relevant bacteria. These enhancements include growth, metabolic fitness, resistance, tolerance to various chemicals, temperature, pH, substrate utilization, and product yield. The evolved strains were developed by growing the target bacteria under controlled laboratory conditions in the presence of specific test chemicals over 100–-1000 generations.[140–142] Whole-genome sequencing and bioinformatics analysis were used to identify the adaptive mutations and deletions responsible for the improved characteristics. In addition to genetic and metabolic engineering, adaptive laboratory evolution of bacterial strains involved in lignin valorization has been found to increase their tolerance to LDACs, eventually enhancing their degradation. Tolerance adaptive laboratory evolution (TALE) of *P. putida* KT2440 by growing it at higher concentrations of *p*-coumaric acid, ferulic acid and in combination for 67 days has resulted in more tolerance, and enhanced degradation by evolved strain than wild type.[143] A recent investigation involved the ALE of *P. putida* ACB122, which expressed *vanAB* genes regulated by P_{tac} promoter, using 20 mM vanillate for approximately 60 days. The resulting evolved strains demonstrated improved growth in vanillin-containing environments characterized by reduced lag phases. Additionally, the introduction of specific amino acid substitutions, P133L, in a LysR-type transcriptional regulator, A24P in vanB reductase, and W15C in FghA hydrolase, led to enhanced growth in minimal medium, where vanillate served as the sole carbon source.[144] Through a combination of genetic engineering and ALE, researchers have developed *P. putida* strains that can utilize non-native substrates to produce valuable compounds.[145,146] A recent study showed that an evolved *Novosphingobium aromaticivorans* JMN123 strain can grow efficiently in the presence of 1 g L^{-1} guaiacylglycerol-β-guaiacyl ether, a lignin dimer. Subsequent whole-genome sequencing and proteomic analyses revealed the involvement of hpvY, a β-HPV-processing enzyme that catalyzes the conversion of β-hydroxypropiovanillone (β-HPV) into vanilloyl acetaldehyde.[147]

Recent developments in automated microbial culture technologies and microfluidic devices have significantly enhanced the efficiency and ease of the ALE.[148,149] These advancements allow the use of various chemicals, real-time monitoring of microbial growth, and precise control over growth parameters, and have made ALE easier and more efficient. Microfluidic devices facilitate droplet-based microbial culturing and advanced liquid handling, allowing compartmentalization of individual cells within water-in-oil droplets or beads for subsequent growth and analysis.[150] The integration of these automated technologies streamlines the experimental workflow and allows us to perform high-throughput screening and simultaneous monitoring of multiple cultures and efficient data collection. This not only accelerates the speed of the entire process but also offers reproducible results and selection of evolved strains.

4.7. Controlling monomeric composition of PHAs

The physical properties of polyhydroxyalkanoates (PHAs), including their melting points and mechanical strengths, are heavily influenced by their monomeric compositions. A significant challenge in producing PHAs from lignin and LDACs is achieving homogeneity, which is critical for downstream applications, market acceptance, and commercial viability. Certain *P. putida* strains, such as B6-2, KT1.5, and KT3-1, can synthesize PHAs

using *p*-hydroxybenzoic acid as a substrate. The resulting PHA primarily consists of 3-hydroxydecanoate (63.1–67.8 mol%), followed by 3-hydroxyoctanoate (14.6–17.2 mol%), 3-hydroxydodecanoate (8–10.1 mol%), 3-hydroxytetradecanoate (7.4–7.6 mol%), and 3-hydroxyhexanoate (1.7–2.1 mol%).[151] In contrast, PHAs produced by *B. cepacia* B1-2 from *p*-hydroxybenzoic acid exhibited a distinct monomeric composition dominated by 3-hydroxybutyrate (98.3 mol%), with minor contributions from 3-hydroxyoctadecanoate (1 mol%) and 3-hydroxyhexadecanoate (0.7 mol%).[151] Similarly, *Halomonas* sp. Y3 has been reported to produce PHAs from LDACs such as vanillic acid, catechol, protocatechuate, and *p*-hydroxybenzoic acid. The resulting polymer consists predominantly of (*S*)-3-hydroxybutyrate (90.25–91.67 mol%) and a smaller fraction of (*R*)-3-hydroxybutyrate (8.33–9.48 mol%).[152] This highlights the variability in PHA monomer composition, depending on the bacterial strain used for production.

A recent study by Wang *et al.*, demonstrated that co-feeding carboxylic acids such as valeric acid, heptanoic acid, and octanoic acid with lignin monomers *p*-coumaric acid and ferulic acid enhanced the monomeric homogeneity of PAHs.[153] Notably, when heptanoic acid was co-fed with octanoic acid and ferulic acid, the engineered *P. putida* Δ*PhaZ*-Δ*aldB*::*PhaC*-Δ*FadBA*, which overexpressed *PhaC*, *PhaJ4*, and *PhaG*, produced PHAs with monomeric compositions of 89.5 mol% and 91.4 mol% 3-hydroxyheptanoate and 3-hydroxyoctanoate, respectively. Under optimal feed-batch fermentation conditions, 2.46 g L^{-1} PHA with 80 mol% 3-hydroxyoctanoate monomer composition was obtained from 12 g of *p*-coumaric acid and 2 g of octanoic acid.[153] Most importantly, the co-feeding of 1 g L^{-1} octanoic acid with 30 g L^{-1} dried corn stover derived alkaline pretreated liquor (APL) resulted in production of 0.93 g L^{-1} of PHAs containing 3-hydroxyoctanoate as a major monomer (85 mol%) followed by 3-hydroxydecanoate, 3-hydroxyhexanoate and 3-hydroxydodecanoate.[153] These encouraging findings underscore the importance of fatty acid precursors for controlling the monomer composition and enhancing production of PHA.

Fermentation temperature significantly influences the monomer composition, thermal properties (including glass transition temperature (T_g) and melting temperature (T_m)), and molecular weight of the produced PHAs. For instance, when *Cupriavidus* sp. L7L was cultivated at 20 °C using levulinate as a carbon source, and the resulting PHAs exhibited a monomer composition of 3-hydroxybutyrate (45.1 mol%), 3-hydroxyvalerate (50.1 mol%), and 4-hydroxyvalerate (4.8 mol%), with a molecular weight of ~4023 kDa. In contrast, at a fermentation temperature of 35 °C, the monomer composition shifted to 3-hydroxybutyrate (66.2 mol%), 3-hydroxyvalerate (30.5 mol%), and 4-hydroxyvalerate (3.3 mol%), with a reduced molecular weight of ~1924 kDa.[154] Substrate type and incubation duration significantly affect the monomeric composition of polyhydroxyalkanoates (PHA). A study involving *Halomonas alkaliantarctica* demonstrated this effect. When cultivated on cheese whey substrate for 24 h, PHA consisting solely of 3-hydroxybutyrate (3HB) monomers was produced. However, extending the incubation period to 72 h resulted in PHA composition of 98.80 mol% 3HB and 1.20 mol% 3-hydroxyvalerate (3HV), respectively. Conversely, when cheese whey mother liquor was used as the substrate, the monomeric composition of PHA remained constant, irrespective of the incubation time.[155] These findings underscore the importance of carefully considering both substrate selection and fermentation duration in PHA production.

Genetic engineering of *Halomonas* sp. Y3 through the expression of *O*-demethylases (*desA* and *ligM*) from *Sphingobium* sp. SYK-6 enabled the biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) with a 3-hydroxyvalerate (3HV) content of 8.44 mol% when syringic acid was supplied as substrate.[156] Further studies have demonstrated that it can produce PHBV with 3HV compositions of 8.48 mol%, 9.11 mol%, and 9.46 mol%, using vanillic acid, protocatechuate, and 4-hydroxybenzoic acid, respectively, as carbon sources.[156] Notably, an engineered *Halomonas* sp. Y3_18 strain co-expressing *O*-demethylases (*desA* and *ligM*) and *p*-hydroxybenzoate hydroxylase (*pobA*) achieved a 3HV content of 7.88 mol% in PHBV when cultivated on a mixed substrate system containing vanillic acid, 4-hydroxybenzoic acid, and syringic acid.[156] Similarly, a recombinant *Halomonas* sp. Y3_coABTS strain overexpressing *O*-demethylases and laccase exhibited a 3HV composition of 6.38 mol%,[156] highlighting that the monomeric composition of PHBV can be fine-tuned based on the intended downstream applications.

4.8. Mining genomes and metagenomes for identification of novel enzymes and pathways

The availability of whole-genome sequences of enormous numbers of microbes, whole metagenome sequence data of samples from diverse environmental niches such as forests, wetlands, farmlands, and marine environments, advanced bioinformatics tools, and machine learning approaches have revolutionised our understanding of microbial lignin degradation. Recent developments in bioinformatics pipelines and open-access online tools have facilitated the rapid screening of metagenomic assembled genomes (MAGs) to identify previously unidentified, unclassified, and uncultured microbes.[157] In a landmark study, Chen *et al.*, demonstrated this potential through a comprehensive analysis of 99 environmental metagenomes.[158] This revealed 474 distinct gene families across the samples involved in lignin depolymerisation, uptake, and catabolism of LDACs.[158] Similarly, the assembly and analysis of genomes from metagenomes enriched with switchgrass-derived alkali lignin revealed the prevalence of numerous enzymes and monolignol degradation pathways, especially caffeic acid degradation pathways, in *Actinobacteria*_BY 70.[159] Although aerobic lignin degradation has been extensively studied, anaerobic processes remain poorly understood. A recent metagenomic study of coastal microbial communities enriched with lignin *in situ* revealed the notable presence of anaerobic bacteria capable of breaking down lignin.[160] This finding suggests that harnessing the unique metabolic pathways of these anaerobic microbes for heterologous expression in industrially important PHA producers could further

enhance PHA production from lignin *via* anaerobic fermentation. Various lignin-degrading enzymes and enzymes involved in the metabolism of LDACs have been identified by mining whole metagenomic data. For example, unique K-type small laccases, O-type laccases,[161] alkaline active feruloyl-CoA synthetases,[162,163] feruloyl-CoA hydratase-lyase,[163] enoyl-CoA hydratase/aldolases.[164] Future studies on the heterologous expression of metagenome-derived efficient enzymes and pathways in industrially important PHA-producing strains to strengthen lignin degradation and the metabolism of LDACs can advance lignin-based PHA production for scalable and sustainable applications.

4.9. Co-culturing approaches for PHA production from lignin

Modern genetic engineering approaches enable the introduction of heterologous lignin-degrading enzymes and pathways into target bacteria to enhance PHA production from lignin. However, the processing of LDACs involves complex enzymatic reactions and cofactors that can strain bacterial metabolism. To address this issue, co-culturing bacteria offers a promising solution by combining complementary metabolic capabilities and mitigating the toxic effects of certain LDACs. Co-culturing lignin-degrading strains with PHA-producing strains efficiently converts aromatic compounds into PHA while broadening the range of metabolizable lignin derivatives. This approach has been practically successful. Co-culturing *Rhodococcus opacus* PD630 and *R. jostii* RHA1 VanA^{-} strains improved lipid production from corn stover lignin compared to single-strain systems.[165] Similarly, the co-cultivation of *Citrobacter freundii* with *Citrobacter* sp.,[166] and *Bacillus subtilis* with *Klebsiella pneumoniae* synergistically enhanced lignin depolymerisation.[167] Notably, co-culturing *Halomonas* sp. Y3_05 (engineered for laccase overexpression) and *Halomonas* sp. Y3_08 (overexpressing the PHA synthesis genes *phaA*, *phaB*, and *phaC*) achieved 1.2 g L^{-1} PHA using alkaline-pretreated liquor, demonstrating its industrial potential. Interestingly, the co-cultivation of *Pleurotus ferulae* and *Rhodotorula mucilaginosa*[168] and white-rot fungi and *R. mucilaginosa* enhanced lignin degradation by modulating the expression of lignin-degrading enzymes.[169] Although there is limited research on developing co-culturing approaches for PHA production from lignin, it holds promise for lignin-based biorefineries. More research is required to make this approach suitable for industrial-scale applications, especially the selection of compatible bacterial strains, the ratio of bacterial strains, and fermentation conditions in conjunction with other engineering approaches.

4.10. Other important factors should be considered for PHA production from lignin

Apart from genetic and metabolic engineering, various other factors also influence PHA production. Briefly, pH, salinity, temperature, carbon–nitrogen (C/N), carbon–phosphate (C/P), dissolved oxygen (DO), fermentation modes (batch, fed-batch, feast-famine, solid state, and continuous), and the addition of supplements greatly modulate PHA production.[170,171] For example, addition of supplements containing ligninolytic enzymes (laccase and aryl alcohol oxidase), mediators (for example 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT)) to enhance the ligninolytic activity of enzymes, dimethyl sulfoxide (DMSO), non-ionic surfactant Tween 80, and silica nanoparticles Aerosol R816 to fermentation medium resulted in the production of 4.5 g L^{-1} of polyhydroxybutyrate from alkaline pretreatment liquor by *Cupriavidus necator*.[89] A recent study demonstrated that supplementing glycerol in the fermentation medium improves the generation of the cofactor $NADP^{+}$ through the TCA cycle. Additionally, the supply of NADPH was improved by chromosomal integration and overexpression of *pntAB*, which encodes a membrane-associated proton-pumping counterpart under the control of the P_{tac} promoter. This optimization significantly enhanced the production of the central metabolite PCA from ferulic acid and *p*-coumaric acid through NADPH, which in turn led to increased production of muconic acid in *P. putida* KT2440.[172] A recent study showed that supplementation of co-precursors such as heptanoic and octanoic acids in the fermentation medium led to1.1 to 2.4 g L^{-1} PHA production by *P. putida* KTYY06 from ferulic acid and *p*-coumaric acid.[153] These studies imply that PHA production from lignin is influenced by the interplay of various factors beyond genetic and metabolic engineering. Hence, it is necessary to integrate engineering, depolymerization, and supplementation strategies to harness maximum PHA yields from lignin.

Notably, the fractionation of lignin from biomass significantly affects its chemical structure, molecular weight, and purity. Industries typically produce two main types of lignin: sulfur-containing lignins, such as kraft lignin, lignosulfonates, and hydrolyzed lignin, and sulfur-free lignins, such as organosolv and soda lignin. These lignins are obtained from various biomasses using various methods, including physical, chemical, physicochemical, and biological processes. In addition, several other techniques have been employed for lignin fractionation, such as ball milling, steam explosion, ionic liquid-based methods, deep eutectic solvent-based methods, enzymatic treatments, and microbial pretreatment.[173,174] Furthermore, numerous research focus is on isolation of lignin from biomass by preserving its native structure to improve quality and depolymerization to produce aromatic compounds for valorization.[175] Despite the availability of various extraction methods, not all types of lignin have been thoroughly assessed as substrates for polyhydroxyalkanoate (PHA) production. Current research primarily focuses on kraft lignin,[176,177] alkaline lignin,[95,111] organosolv lignin,[178] alkaline pretreated liquor,[95,105,111,151,153,179,180] and lignin-containing streams[113,181,182] for PHA synthesis. Among the types of lignin used when alkaline pretreated liquor was used as the substrate, more PHA production was observed (Table 1). Liu *et al.* (2021) has developed "plug-in-processes of lignin" to produce PHA using an engineered *P. putida* KT2440 from ammonia fiber expansion lignin, dilute sulfuric acid pretreatment lignin, steam explosion pretreatment lignin, liquid hot water pretreatment lignin and sodium hydroxide pretreatment lignin (Table 1) and the findings showed highest PHA production

(ranging from 2.2–4.5 g L^{-1}).[61] These studies highlight the applicability of alkaline-pretreated liquor and pretreated lignin as efficient substrates for PHA production, making them promising options for further research and development.

Research has shown that alkali sterilisation improves lignin degradation, promotes the growth of *Rhodococcus opacus* PD630, and increases lipid production compared with conventional autoclave-based sterilisation.[183] This process involved increasing the pH of the kraft lignin-containing growth medium to 12.7 using NaOH, and then incubating at 180 rpm for 24 h. This incubation facilitated the complete solubilisation of lignin and sterilisation of the medium. After incubation, the pH of the medium was aseptically adjusted to 7.2 using HCl, and used for lipid production using *R. opacus* PD630. Subsequent analysis revealed that this sterilisation strategy reduced the molecular weight of kraft lignin and the formation of colloidal lignin particles, leading to greater lignin degradation and the utilisation of LDACs for lipid production by *R. opacus* PD630.[183] Similarly, a non-sterilised fermentation method was developed to produce PHA from lignin and LDACs This process is suitable for alkali-halophilic bacteria such as *Halomonas* sp. Y3, and *Halomonas alkalicola* M2 which can grow at high NaCl concentrations (ranging from 10–150 g L^{-1}) and alkaline pH (ranging from 8–12).[95,152,184] Under these conditions, the most common contaminating bacteria do not grow, which enables us to skip the conventional sterilisation process. Optimum lignin degradation and PHA production by *Halomonas* sp. Y3 and *H. alkalicola* M2 were observed at pH 9.0 and 60 g L^{-1} NaCl and pH 10.0 and 50 g L^{-1} NaCl, respectively. The development of non-sterilization-based fermentation methods is expected to reduce the energy-intensive sterilization steps and requirements of facilities to maintain aseptic conditions. The implementation of these alternative sterilisation and non-sterilisation-based methods in biorefineries will not only enhance the lignin degradation efficacy and PHA production, but will also provide substantial economic benefits by reducing the energy consumption for sterilisation and the requirements of facilities to maintain aseptic conditions in bioproduction processes.

4.11. Prospects for using yeasts for bioconversion of lignin and LDACs into PHA

Yeasts are widely accepted and versatile microbial cell factories for the industrial production of therapeutic proteins, enzymes, bioactive compounds, and biofuels.[185,186] *Saccharomyces cerevisiae* is an extensively studied model, alongside other species such as *Pichia pastoris*, *Hansenula polymorpha*, *Yarrowia lipolytica*,[187,188] *Kluyveromyces lactis*, *Candida glabrata*, and *Candida utilis*.[189] While wild-type strains do not naturally produce PHA, and genetic engineering to express bacterial PHA synthases has been shown to enable PHA production in *S. cerevisiae*.[190–192] However, researchers have identified that wild-type yeasts with natural PHA-producing potential, including *Rhodotorula minuta*,[193] *Pichia kudriavzevii*,[194] and *Hanseniaspora valbyensis*.[195] Chromosomal integration of PHA biosynthesis genes (*PhaA*, *PhaB1*, and *PhaC1*), genes encoding cellobiose-degrading enzymes derived from *Neurospora crassa*, and extrachromosomal expression of cellodextrin transporter gene CDT-1 derived from *N. crassa* have enabled PHA production from cellulosic substrates.[196] Similarly, engineered *Rhodotorula glutinis* expressing *PhaA*, *B* and *C* genes from *C. necator* has been reported to produce 2.87 g L^{-1} PHA using glycerol as carbon source.[197] Further research has shown that the monomeric composition of PHA can be altered by expressing different types of PHA synthase genes.[192]

A cold-adapted *Rhodosporidiobolus colostri* has been reported for its ability to degrade and utilize LDACs, such as ferulic acid, *p*-coumaric acid, and 4-hydroxybenzoic acid, at low temperatures.[198] Similarly, *Cutaneotrichosporon oleaginosus* contains *ortho*-cleavage pathways to metabolize phenol, 4-hydroxybenzoate, and resorcinol and produce lipids.[199,200] *Rhodotorula mucilaginosa* can grow on a medium containing 90% liquor derived from base-catalyzed depolymerization of a lignin-rich stream from corn stover.[201] It is important to note that researchers have engineered *S. cerevisiae* to utilize LDACs to homoeriodictyol,[202] protocatechuic acid,[203] vanillin,[204] and fraxetin.[205] Briefly, in a recent study, researchers have engineered *S. cerevisiae* by knocking out phosphoglycerate mutase and chorismate mutase, integrating *de novo* biosynthetic pathways into its chromosome to transform mixture of lignocellulosic biomass derived sugars and aromatic compounds into vanillin.[206] These advances highlight that further integration of *ortho*- or *meta*-cleavage pathways in yeasts can funnel LDACs into PHA production.

Since, yeasts such as *S. cerevisiae*, *P. pastoris*, *Pichia methalonica*, *K. lactis*, and *Y. lipolytica* are widely used as expression hosts for the heterologous production of lignin depolymerising enzymes such as Lac, MnP, LiP, and VP.[207] Optimising enzyme secretion pathways and integrating aromatic funnelling pathways into these strains could enable simultaneous lignin depolymerisation and PHA synthesis. Compared to bacteria, yeast cells are larger in size and can be separated from the fermentation medium by sedimentation which can reduce the cost of downstream processing. Yeasts, especially *S. cerevisiae*, are generally regarded as safe (GRAS), and PHA derived from yeast requires fewer purification steps for food and biomedical applications. Considering these advantages, well-developed genetic and metabolic engineering tools, membrane transport systems, protein secretory pathways, and proven industrial-scale production processes,[208,209] future studies should focus on utilising yeast-based cell factories for the bioconversion of lignin and LDACs into PHA. This may pave the way for efficient lignin valorization and cost-effective production of PHA.

5. Challenges in purification of PHA

Although the production of PHA from lignin and lignin depolymerization-derived aromatic compounds is viewed as an economical approach, the extraction of PHA from microbial biomass is associated with a high cost. The typical extraction of PHA involves several key steps. 1. Biomass separation: collection of microbial biomasses from the fermentation broth

through centrifugation, filtration, or sedimentation. 2. Cell lysis, removal of non-cellular PHA mass (NCPM) and PHA recovery: pretreatment is necessary to lyse the cells and release the PHA granules. Various techniques, including heating, freezing, ball milling, sonication, high-pressure homogenization, and chemical treatments using sodium hypochlorite, detergents, non-ionic surfactants, or proteases, are used. 3. PHA separation: after cell lysis, PHAs are recovered from the lysate by liquid–liquid extraction using chlorinated solvents or solvents, precipitation, filtration, sedimentation, *etc*. 4. Purification of PHA: the steps involved redissolution of PHA, bleaching by H_2O_2 or ozone to deodorise, and removal of residual contaminants.[170,210,211] Based on the intended applications of PHA, the purification steps may vary. To facilitate the replacement of conventional plastics with PHA-based materials, a reduction in the cost of PHA extraction and the implementation of ecofriendly operation is required. As a result, more attention has been paid to the development of economical and environment-friendly extraction methods by minimizing the use of detergents and solvents and opting for less toxic solvents. At the same time, the non-cellular PHA mass (NCPM) should be removed completely from the PHA, as it exhibits immunogenicity. Recently, many eco-friendly methods have been developed for PHA extraction and purification. For example, PHA was extracted from *Methylosinus trichosporium* OB3b using sodium hypochlorite for cell lysis, extraction using 1,3-dioxolane, followed by precipitation of PHA by methanol as an antisolvent, resulting in purity ranging from 99.2–99.4% and recovery rate ranging from 85.5–92.1%.[212] In a recent report, researchers have demonstrated that application of crude protease extract from *Aspergillus oryzae* for breaking *Paraburkholderia sacchari* cells followed by extraction with 1,3-dioxolane for 6 h at 80 °C can extract poly(3-hydroxybutyrate) with high recovery rate and purity.[213] Heterologous expression of nuclease in *Cupriavidus necator* and *Delftia acidovorans* reduces the viscosity of the cell lysate, which is one of the factors necessitating the addition of more chemicals or detergents for cell lysis.[214] Future studies on the application of protease and nuclease for simultaneous lysis of cells with reduction in viscosity could improve PHA recovery.

At the industrial scale, obtaining high-grade PHA involves several key steps. First, acidification of cells containing PHA at pH ≤ 6.0, followed by high-pressure homogenization (500–1500 bar) to lyse the cells, and alkalisation of the cell extract by adding sodium hydroxide/potassium hydroxide to increase pH ≥ 9.0. After alkalisation, sodium dodecyl sulphate was added, and the cell suspension containing PHA was diluted with water (1 : 3). Then, the PHA suspension was concentrated by tangential filtration, and a flocculating agent was added before orthogonal filtration to obtain purified PHA. This method was developed by Bio-On (Italy) and used for large-scale purification of PHA.[215] To remove lipids and other contaminants from the microbial cells, cells were washed with ethanol (a class III solvent that is considered to have low toxicity to humans) at room temperature and air-dried to remove residual ethanol. PHA was then extracted from the ethanol-washed and dried cells using an organic solvent (preferably acetone, which is less toxic to humans). Ethanol was used to precipitate PHA from the organic extract at room temperature, followed by centrifugation or filtration to collect the PHA and drying under vacuum, air, or desiccants. Resulting PHA can be used for manufacturing medical implants.[216] Venvirotech Biotechnology Sl has developed a simplified method for PHA extraction from bacterial cells. This process requires a minimal quantity of sodium hypochlorite for cell lysis, followed by washing with water to eliminate water-soluble contaminants from bacteria and sodium hypochlorite. This was followed by PHA extraction using dimethyl carbonate (low toxicity and high biodegradability) at 90 °C for 1 h with intense stirring. The final step involves concentrating the PHA-containing dimethyl carbonate fraction by rotary evaporation and precipitation of PHA through the addition of cold methanol.[217] A chlorine-free method for PHA extraction has been developed, which involves acidifying microbial biomass, cell lysis using a combination of NaOH + H_2O_2, and ethyl acetate extraction to recover PHA from biomass.[218] However, this method requires a high temperature (115 °C) and pressure (300 kPa), raising concerns about its industrial viability due to potential power consumption. It is presumed that the use of solvents such as acetone or dimethyl carbonate may help reduce power consumption during the extraction process.

Reducing costs related to PHA production and downstream processing, as well as minimising the environmental impact, are essential. Additionally, it is crucial to completely eliminate the NCPM from the extracted PHA because any residual NCPM may trigger immune responses. Overcoming these challenges will pave the way for economically viable PHA production and facilitate the transition from conventional plastic dependence to PHA-based biodegradable plastics in various industries. The pilot-scale production of PHAs from lignin or LDACs remains underdeveloped. While established methods for PHA extraction can be applied to extract PHA produced by microbes using these carbon sources, an additional washing step is necessary to remove residual aromatic compounds and unutilized lignin. We propose that alkalizing the fermentation media to a pH of 9.0 or higher (most lignin is soluble at alkaline pH) before collecting the bacterial biomass could effectively solubilize lignin aggregates and cell-bound lignin. This should be followed by an ethanol wash to eliminate lipids and other contaminants before the recovery step. This approach may enhance the overall efficiency of extraction and the purity of the PHA produced from lignin-based substrates.

6. Techno-economic analysis (TEA) of lignin to PHA conversion

Although the abundance and low cost of lignin and lignin-rich streams from biorefineries make it an attractive renewable substrate, PHA production from lignin is still in the early stage of research, and most of the studies are limited to the laboratory scale (Tables 1 and 2). Consequently, unlike PHA production from standard carbon sources, comprehensive TEA for the microbial transformation of lignin to PHA has not yet been

conducted. TEA revealed that PHA production using citric molasses as a carbon source by *Cupriavidus necator*, pretreatment of bacterial biomass either by high-temperature (95 °C for 45 min) or high-pressure homogenisation (90 MPa), followed by propylene carbonate extraction at a production cost of $ 4.28 per kg. However, with high cell density fermentation (140 g L^{-1}) and further scaling up of production, the cost could be reduced to $ 2.71 per kg when the annual PHA production reaches 10 000 kg.[219] Similarly, TEA showed PHA production by *Ralstonia eutropha* using carob pod extract in an annular bioreactor, and the collection of PHB-containing biomass by ceramic membrane separation offered an annual production of 30 267 tons with an annual revenue of 509.37 million $ and a pay-out period of 4.8 years. Furthermore, this study revealed that the utilisation of carob pod extract accounted for 14–16% of the production cost, whereas using purified sugars as substrates accounted for 67% of the total production cost.[220] This suggests that cheaper substrates are crucial for reducing overall production costs. Similar to the cost of substrates, reduction of cost associated with PHA extraction equally reduces the production cost substantially. For example, Rajendran and Han demonstrated that the minimum selling price of PHA produced by *Enterobacter aerogenes* using food waste hydrolysate was found to be 4.83 $ per kg, when the increasing biomass load to 30% and decreasing chloroform usage during PHA extraction has lowered minimum selling price to 2.41 $ per kg.[221] In the case of lignin-PHA production, TEA analysis evaluated plug-in processes for producing PHA by *P. putida* using soluble lignin streams derived from corn stover, and various pretreatment methods in fed-batch mode in shake flask fermentation were examined.[61] Briefly, soluble lignin streams from ammonia fibre expansion, steam explosion, hot water, sulfuric acid, and NaOH pretreatments were used as substrates for PHA production. Among these, lignin from ammonia fiber expansion yielded the lowest estimated minimum selling price for PHA at $6.18 per kg, followed by steam explosion ($6.82 per kg), liquid hot water ($8.35 per kg), sulfuric acid ($9.58 per kg), and sodium hydroxide ($11.99 per kg) pretreatments.[61] Although these prices currently exceed those of PHA derived from conventional substrates, scaling up production, optimising fermentation conditions, enhancing bacterial growth on lignin, and applying laccase pretreatment to lignin streams could reduce production costs and improve economic viability. Ammonia fibre expansion lignin, in particular, shows promise for further development owing to its competitive baseline cost. Additionally, future studies are required to evaluate the TEA of various lignin depolymerisation methods, the separation of LDACs, and their use as carbon sources for PHA production.

7. Conclusion and future perspectives

The microbial conversion of lignin into PHA offers a promising opportunity for sustainable bioplastic production, potentially addressing an alternative solution for mitigating the environmental concerns associated with conventional plastics. Although extensive research and development has focused on the isolation of lignin from biomass, depolymerization to produce aromatic monomers, and their conversion into PHA using microbes, several challenges still need to be addressed to achieve industrial-scale implementation and ensure economic viability. First, it is important to develop eco-friendly lignin isolation methods and improve the available methods to preserve the native structure or minimize the structural modifications of lignin, which is essential for subsequent depolymerization and microbial bioconversion into PHA. Enhancing depolymerization efficiency and monomer selectivity is another vital area that requires further research and development. Current methods for lignin depolymerization yield a heterogeneous mixture of aromatic compounds, many of which are difficult to metabolize or not readily metabolized by microbes. Hence, improving the monomer selectivity of depolymerization to produce a narrow range of aromatic compounds would significantly improve PHA production. Many microbes do not grow well using LDACs; therefore, it is necessary to enhance the tolerance and growth of industrially important PHA-producing strains. Genetic, metabolic, and protein engineering approaches coupled with adaptive laboratory evolution have shown potential in developing robust and efficient microbial chassis capable of biotransforming LDACs into PHA in industrially relevant yields. While numerous bacteria have been documented to produce PHA, research on PHA production from lignin and LDACs has been mainly centred on *P. putida* KT440, with few studies on *Halomonas* species, and other bacteria. Hence, future research should be broadened to include other important bacteria and yeasts that produce PHA, which could potentially enhance the efficiency of PHA production from lignin. Developing economic and ecofriendly downstream processing of PHA based on the desired application is vital for the commercial viability and environmental sustainability of the microbial transformation of lignin to PHA. Currently, many PHA extraction methods depend on detergents, solvents, and energy-intensive processes, which compromise the overall sustainability of production. However, recent developments in PHA extraction have demonstrated the possibility of using mechanical methods for cell lysis and green solvents for PHA extraction and purification. Further research is required to improve the economic efficiency of this process and minimize chemical usage. Continued research on lignin fractionation, depolymerization, strain development, fermentation strategies, and downstream processing is crucial for producing PHA from lignin as a promising alternative to conventional plastics. In addition, it is necessary to conduct comprehensive life cycle assessments to understand the environmental impact of PHA production using lignin- and LDACs compared to conventional petroleum-based plastics and other bio-based plastic alternatives. Continued research on lignin fractionation, depolymerization, strain development, fermentation strategies, and downstream processing is crucial for producing PHA from lignin as a promising alternative to conventional plastics. Ultimately, the successful development

of industrial-scale production of PHA using lignin could contribute to circular bioeconomy and reduce dependence on conventional plastics. In addition, it will maximize the utilization of lignocellulose biomass and provide additional economic benefits to biorefineries.

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

Conflicts of interest

There are no conflicts to declare.

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