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Lignin valorization by bacterial genus *Pseudomonas*: State-of-the-art review and prospects

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Abstract

The most prominent aromatic feedstock on Earth is lignin, however, lignin valorization is still an underrated subject. The principal preparatory strategies for lignin valorization are fragmentation and depolymerization which help in the production of fuels and chemicals. Owing to lignin's structural heterogeneity, these strategies result in product generation which requires tedious separation and purification to extract target products. The bacterial genus *Pseudomonas* has been dominant for its lignin valorization potency, owing to a robust enzymatic machinery that is used to funnel variable lignin derivatives into certain target products such as polyhydroxyalkanotes (PHAs) and cis,cis-muconic acid (MA). In this review, the potential of genus *Pseudomonas* in lignin valorization is critically reviewed along with the advanced genetic techniques and tools to ease the use of lignin/lignin-model compounds for the synthesis of bioproducts. This review also highlights the research gaps in lignin biovalorization and discuss the challenges and possibilities for future research.

Keywords: Lignin biorefinery; Biovalorization; Metabolic engineering; Pseudomonas; Enzymatic conversion.

1. Introduction

Lignin constitutes 15–40% dry weight of most plants and is a profuse biological macromolecule along with hemicellulose and cellulose (**Cao et al., 2019, 2017; Schutyser et al., 2018**), it is an important but significantly underrated industrial feedstock. In 2017, approximately 130 million tons of kraft pulp were produced worldwide, which generated 70 million tons of

lignin alongside (**Supanchaiyamat et al., 2019**). Pulp and paper industries are significant sources of lignin but only 5% of this waste lignin is combusted for the generation of power and heat (**Cao et al., 2018**). Regardless of a biogenic origin, the combustion of lignin is responsible for the generation of air pollutants such as particulate matter and aromatic compounds (e.g., styrene, benzene) (**Corona et al., 2018**). Studies have concluded such releases to be amongst the most oppressive type of air pollution from lignin biorefinery operations.

In recent years, different catalytic strategies and thermochemical transformation have been proposed to transform lignin into valuable products such as biooil, gaseous hydrocarbon, phenol, and cresol via oxidation (**Dai et al., 2018; Cao et al., 2019)**, liquefaction (**Matsagar et al., 2019**), hydrolysis (**Bhagia et al., 2016)**, hydrogenolysis (**Fang et al., 2019)**, carbonization (**Ruan et al., 2018**), pyrolysis (**Nie et al.,2020; Liu et al., 2018; Kumar et al., 2020b**), and gasification (**De Blasio et al., 2019)**. These processes have advantages such as improved conversion efficiency, high yield, high throughput, and operation flexibility (**Cao et al., 2019; Fan et al., 2018; Anthony et al., 2019**). Nevertheless, these processes are not considered fully compatible with the environment due to their limitations including large water consumption, high energy demand, process complexity, and often low selectivity (**Cao et al., 2019; Lu et al., 2018; Fan et al., 2018**). Lignin processing consists of two stages: (a) polymerized lignin is cleaved to generate subunits, i.e., depolymerization; and (b) subunits are assembled to generate value-added products, i.e., upgrading (**Anthony et al., 2019**). They can be accomplished through catalytic, biological, and thermochemical processes which are followed by extensive purification and separation which may or may not use a biological catalyst (enzyme/microbe) to funnel lignin and its compounds to a single target product (**Becker and Wittmann, 2019; Corona et al., 2018**). A competent lignin valorization strategy involving both depolymerization and upgrading

66 stages, along with separation and purification, can attain environmental sustainability and
67 economic viability for widespread industrial applications (**Wu et al., 2018**).

68 The microbial conversion of lignin to value-added products such as PHAs (**Kumar et al.,**
69 **2018)** and fatty acid (**Wang et al., 2019**) is considered as an environmentally friendly and cost-
70 effective approach (**Xu et al., 2018a**). Many recent studies emerged and described the
71 application of potential microbial strains in the process of lignin biovalorization (**Barton et al.,**
72 **2018; Kohlstedt et al., 2018**). Lignin valorization follows variable pathways to produce
73 chemicals of commercial interest (up to pilot scale) such as MA, adipic acid (AP), and
74 terephthalic acid (TA) (**Kohlstedt et al., 2018; Vardon et al., 2015**). In comparison to the
75 existing second-generation biorefineries, biological conversion of lignin/lignin-model
76 compounds is a sustainable green alternative to traditional fossil routes (**Corona et al., 2018**).
77 Beyond energy applications, it is a cost-friendly option with the capability to reduce greenhouse
78 gases (GHGs) emissions (**Corona et al., 2018**). It also holds applications in industrial chemical
79 production, for example, MA, AP, pyruvate, lactate, and PHAs, thus presenting an overall win-
80 win situation (**Wang et al., 2018; Vardon et al., 2016; Linger et al., 2014**) **(Table 1)**.

81 Earlier report has suggested effective lignin metabolization by brown and white-rot fungi;
82 however, fungi as biological catalysts have not been efficiently commercialized owing to a
83 difficulty in recombinant protein expression and complex genome (**Bugg et al., 2011**). Due to a
84 unique "biological funneling" route, bacterial strains have started gaining attention in lignin
85 valorization using lignin as the substrate for production of targeted products (**Xu et al., 2019a;**
86 **Kumar et al., 2018; Linger et al., 2014**). Bacteria strains depolymerize the lignin into simple
87 monomeric forms such as *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (**Grabber**
88 **et al., 2019**), Subsequently, these monomeric units are converted into protocatechuate,

89 catechuate, and vanilaate via bacterial lignin funneling pathway (**Lin et al., 2019**). Finally, these
90 lignin derivatives are metabolized by bacteria for its cell growth and biosynthesis of value-added
91 products such as PHAs (**Wang et al., 2018; Linger et al., 2014**), MA (**Sonoki et al., 2018;**
92 **Kohlstedt et al., 2018**), muconolactone (**Okamura-Abe et al., 2016**), etc. Other advantages of
93 applying a bacterial strain include small genomic size, possible genetic manipulations, ease of
94 recombination and gene expression (**Kumar et al., 2019**). Recent studies have included potential
95 bacterial strains in the degradation of heterogeneous compounds, lignocellulosic biomass (LCB)
96 pretreatment and liquor sludge treatment (from pulp and paper industry) (**Salvachúa et al., 2020;**
97 **Majumdar et al., 2019; Zhuo et al., 2018; Hooda et al., 2018**).

98 Among the microbial domains, lignin depolymerization is highly favored by genus
99 *Pseudomonas* with significant relevance to industrial biotechnology. *P. putida* is especially
100 recognized for its capability to process aromatic compounds through biochemical routes
101 (**Salvachúa et al., 2020; Lee et al., 2019; Corona et al., 2018; Nikel and de Lorenzo, 2018**).
102 Bacterial genus *Pseudomonas* falls in the *Pseudomonads*/γ-*proteobacteria* taxon and bears the
103 potential of being a catalyst that can generate valuable products from lignin and carbohydrate
104 fraction of lignocellulosic waste (**Ghosh et al., 2019; Lee et al., 2019; Ravi et al., 2018**). In the
105 most promising field of bacterial conversion of lignin into valuable products, the application of
106 genus *Pseudomonas* is critically scrutinized in this review. The implementation of advanced
107 genetics tools and technologies and its state-of-the-art are discussed. Also, the research gaps in
108 lignin biovalorization along with challenges and prospects are explored for future research.

109 **2. Extraction of lignin for chemicals and fuels**

110 Extraction of lignin from LCB and maintaining its high-grade purity hold a significant
111 position in biovalorization. Lignin extraction is a challenging process due to the strong covalent

bonding of the lignin-cellulosic complex (LCC) along with hemicellulose constituents (glucomannan and xylan) of lignin (**Ragauskas et al., 2014**). The chemical structures of lignin involve methoxyl, carboxyl, carbonyl, and hydroxyl groups which constitute its three basic monomers sinapyl, p-coumaryl, and coniferyl alcohol known as monolignols (**Bajwa et al., 2019**). Lignin is prone to bond breakage during extraction, which demands extra attention to obtain uniform structural lignin. Before extraction, carbohydrate fraction is disengaged from lignin by breaking LCC bonds followed by depolymerization, which decreases the size of metabolized entities, increases their solubility, and dissociates them from the biomass. However, lignin has an active tendency to repolymerize at a faster rate, which leads to the synthesis of its intermediates in between the process (**Dikshit et al., 2020)**. There have been various laboratories and industrial-scale processes of lignin extraction from LCB noted so far, which have been discussed in recent articles (**Hassan et al., 2018; Bhagia et al., 2016; Wang et al., 2019; Schutyser et al., 2018)**.

3. Conversion/depolymerization of lignin

Lignin is prominently released as a liquor waste in paper industries and LCB fermentation, which can be used as feedstock to generate chemicals and renewable fuels (**Wu et al., 2018**). Lignin depolymerization is challenging due to its stern recalcitrant behavior, active tendency of intermediates to proceed secondary reactions, and its structural diversity (**Dikshit et al., 2020**). Lignin feedstock decides the type of compound generation depending on the molecular and chemical structure of lignin (**Becker and Wittmann, 2019**). The type of conversion technology employed also decides the solid/liquid/gas phase of the generated products. The key parameters for conversion technologies include the processing conditions (e.g., temperature, pressure, chemical concentration, etc.) (**Kucharska et al., 2018**) along with the performance metrics (e.g.,

throughput rate, composition, product yield, selectivity, etc.) (**Xu et al., 2019b**). There have been several lignin conversion technologies reported so far, which include thermochemical and catalytic conversion such as oxidation (**Cao et al., 2019)**, pyrolysis (**Liu et al., 2018**), carbonization (**Ruan et al., 2018**), gasification (**De Blasio et al., 2019**), biological/enzymatic conversion (**Salvachúa et al., 2020; Schutyser et al., 2018; Wang et al., 2018**), and hybrid conversion (**Wu et al., 2017; Beckham et al., 2016**). The thermochemical and catalytic conversion technologies of lignin valorization are beyond the scope of the current review; the remaining applicable conversion technologies are discussed below.

3.1 Biological/enzymatic conversion

In view of the inherent challenges associated with the thermochemical conversion technologies (**Schutyser et al., 2018**), biological (bacteria, fungi, etc.), and enzymatic conversion technologies of lignin are considered as more significant and eco-friendly (**Ponnusamy et al., 2019**). Recent reports signify the importance of microbial conversion of lignin into valuable products with single (pure) strains (**Salvachúa et al., 2020; Kumar et al., 2018; Wang et al., 2018**) and with microbial consortia (mixed strains) (**Bilal et al., 2018; Wu and He, 2013**). Depolymerization of lignin via biocatalysis involves several enzymes, for example, in the enzymatic degradation of β-aryl ether, biphenyl bond, and so on (**Xu et al., 2020**). Additionally, several bacteria strains were screened and characterized for the utilization of lignin as a carbon source for producing bioplastics, biofuels and bio-based chemicals (**Xu et al., 2020; Wang et al., 2018**). Biological systems function well in mild conditions, which can reduce the cost incurred by maintaining higher pressure and temperature (**Anthony et al., 2019**). However, these processes are time-consuming and incur extra maintenance/operation cost, which are the major challenges in commercialization of bioprocessing technologies (**Xu et al., 2019b**).

158 The search for an efficient enzyme may overcome the existing limitations. For example, laccases
159 and peroxidases are naturally produced by white-rot fungi, which, however, follow complex
160 cultivation and biosynthetic pathways, rendering the commercial generation of a fungal enzyme
161 uncertain and ambiguous (**Salvachúa et al., 2020; Li et al., 2019; Xu et al., 2019b; Wang et**
162 **al., 2018**). Lignin-degrading enzyme also presents another challenge owing to its poor solubility
163 in the bioprocessing media (**Constant et al., 2016; de Gonzalo et al., 2016**). To overcome the
164 challenges associated with the thermochemical and biological conversion technologies of lignin,
165 the concept of hybrid technologies has been proposed.

166 ***3.2 Hybrid conversion technologies***

167 Each technology adopted to date has its pros and cons, so there comes a hybrid approach to
168 combine the advantages of biological conversion and thermochemical technologies (**Wu et al.,**
169 **2017**). The reaction kinetics and catalytic conversion of the thermochemical approach are
170 merged with microbial funneling of biological approach for lignin depolymerization and down
171 streaming, respectively (**Beckham et al., 2016**). The biological down streaming will help in
172 achieving efficient recovery of generated products of depolymerized lignin with high selectivity
173 (**Anthony et al., 2019**), which can occur through various microbial metabolic pathways. The
174 biological funnel was loaded with metabolic intermediates including catechol and
175 protocatechuate, followed by their conversion to central metabolites such as acetyl-CoA, which
176 eventually facilitated the selective production of target compounds (**Wang et al., 2018; Chen**
177 **and Wan, 2017**). The hybrid technologies have mainly been applied for the utilization of sugar
178 (**Schwartz et al., 2014; Sheldon, 2014)**. For example, cellulosic conversion technologies
179 involved the thermochemical depolymerization of saccharide (**Taherzadeh and Karimi, 2008**)
180 followed by the generation of pyrolytic sugars (**Rover et al., 2014**), which were eventually

181 converted to valuable bioethanol via microbial fermentation (**Schwartz et al., 2014**). LCB's
182 sugar conversion via hybrid technology is a significant research area; however, lignin and lignin
183 aromatics demand more attention towards its valorization via hybrid technology (**Salvachúa et**
184 **al., 2020; Kumar et al., 2018; Wang et al., 2018**).

185 Studies on hybrid technology have demonstrated integration of the thermochemical process
186 with a microbial catalyst to metabolize aromatic compounds (**Linger et al., 2014**). However, the
187 main objective of these studies was for the optimization of LCB pretreatment, separation, and
188 recovery of sugar and lignin. To achieve an efficient lignin conversion via hybrid technology,
189 many essential parameters should be considered, including the separation of soluble lignin
190 breakdown products, reduced inhibitors generation, optimized microbial growth for better yield
191 and selectivity, and process design for better yield at commercial scale (**Anthony et al., 2019**).
192 Pretreatment techniques such as ammonia fiber expansion, alkaline pretreatment, etc., have been
193 developed for LCB to highlight the potency of hybrid conversion processes (**Lin et al., 2016;**
194 **Salvachúa et al., 2015; Linger et al., 2014**). The chemical pretreatments will facilitate
195 depolymerization of lignin into more soluble oligomeric forms (simple forms), which are
196 simultaneously utilized by microbes for the production of fuels and chemicals (**Xu et al., 2020**).
197 This hybrid technology could be a plausible, cost-effective, and greener approach, but it also
198 requires robust microbial strains, which improvise the biovalorization of lignin and lignin
199 derivatives. In this context genus *Pseudomonas* could play a tremendous role.

200 **4. Why *Pseudomonas*?**

201 Genus *Pseudomonas* are well known for secretion of oxidative enzymes such as
202 oxidoreductases, PpDyP, and dioxygenase to induce the degradation of lignin and generation of
203 lignin oligomers such as β-aryl-ether and coniferyl aldehyde (**Wang et al., 2018**). These

generated oligomers are catabolized via aromatic compounds funneling pathways (**Salvachúa et al., 2020; de Gonzalo et al., 2016; Rahmanpour and Bugg, 2015**). Additionally, they can easily acclimatize to the environment and are easy to be genetically modified (**Nikel and de Lorenzo, 2018; Lin et al., 2016**). These characteristics indicate the significance of genus *Pseudomonas* in the biovalorization of lignin and lignin derivatives. **Ravi et al. (2018)** cultivated the *P. deceptionensis* (DSM 105530) bacterial strain isolated from the sediments of the Baltic Sea in minimal salt media (MSM), having 5 mM lignin model compounds such as ferulate, p-coumarate, benzoate, syringate, vanillin, guaiacol, vanillate, 4HB, and vanillyl alcohol (VA) separately at 27 °C and 180 rpm with initial optical density (OD) 0.5. The bacterial strain *P. deceptionensis* (DSM 105530) showed the maximum specific growth (0.3 h^{-1}) for benzoate and minimum specific growth (0.12 h^{-1}) for VA. Previously **Ravi et al. (2017)** cultivated the *P. putida* KT2440 in MSM supplemented with 5 mM vanillin, vanillate, 4HB, *p*-coumarate, benzoate, and ferulate separately or in mixed form at 28 °C and 170 rpm. *P. putida* KT2440 significantly utilized the six aromatics as carbon source. *P. putida* KT2440 showed the maximum specific growth (0.27 h^{-1}) for benzoate and minimum specific growth for ferulate (0.21 h^{-1}). The above studies showed the growth of bacterial strain *Pseudomonas* using various lignin model compounds. In spite of the large number of investigations related to the utilization of aromatic as a carbon source by *P. putida* via β-ketoadipate rout, some key parameters such as the kinetics and flux capacities are still not well understood.

P. putida has a specific *β*-ketoadipate rout for the use of monomeric lignin derivatives as the sole carbon and energy source for the production value-added products (**Wang et al., 2018**). The *β*-ketoadipate rout could be separated into two different branches based on their end products. In one branch, lignin model compounds such as *p*-cresol and 4HB as well as lignin derivatives such

as vanillate and coniferyl alcohol were transformed to protocatechuate, whereas the second branch transformed aromatic hydrocarbons, aromatic amines, and monomeric lignin to catechol (**Chen and Wan, 2017**). The end products of both branches (protocatechuate and catechol) were further transformed to β-carboxymuconate and muconate facilitated by enzymes protocatechuate 3,4-dioxygenase and catechol 1,2-dioxygenase, respectively (**Dikshit et al., 2020**). Furthermore, these aromatic products, β-carboxymuconate and muconate, could be converted to succinyl-CoA and acetyl-CoA via several enzymatic routes (**Figure 1**). Afterward, enzymes such as fatty acid synthase II and acetyl-CoA carboxylase as well as other metabolic routes can use these final products as substrates for the production of value-added products such as PHAs (**Wang et al., 2018**).

Several recent studies recommended the use of genus *Pseudomonas* in biovalorization of lignin at industrial level (**Salvachúa et al., 2020; Nikel and de Lorenzo, 2018; Wang et al., 2018**). An array of Dyp-type peroxidases was investigated in *P. fluorescens* Pf-5, which revealed enzyme Dyp1B as lignin oxidizer (**Rahmanpour and Bugg, 2015**). There are other competent strains including *Pseudomonas* sp. PKE117 in comparison to white rot fungi as mentioned by **Yang et al. (2007)**, which are capable of softwood bio-pulping and ensure to decompose benzene rings and destroy guaiacyl propanoid lignin units. **Linger et al. (2014)** realized that the ability of lignin depolymerization by *P. putida* KT2440 was attributed to the analytical structure of the produced medium chain length-PHA (mcl-PHA) using alkali-pretreated liquor (APL) supplemented with ^{13}C-labeled carbon. *P. putida* mt-2, a mega plasmid was detected in the strain KT2440 and its parental copy. Gel permeation chromatography (GPC) and Klason lignin analysis concluded that the plasmid was responsible for reducing a significant fraction (30%) of high molecular weight (HMW) APL in 7-day culture (**Salvachúa et al., 2015**). Similarly, strain

P. putida NX-1 and *P. putida* A514 chosen from fifteen screened strains demonstrated lignin modification and optimized growth in a minimal salt medium (MSM) with 1% lignin (alkali-insoluble) (**Xu et al., 2018b; Lin et al., 2016**). SEM (scanning electron microscopy) and FTIR (Fourier transform infrared spectroscopy) were applied to analyze the morphological and chemical structural changes (decreased number of guaiacyl unit and particle size) in lignin induced by strain *P. putida* NX-1 (**Xu et al., 2018b**).

Another successful attempt was made to degrade alkaline lignocellulosic material by *Pseudomonas* strain Q18 extracted from rotten wood (**Yang et al., 2018**). This strain could utilize lignin efficiently in the presence of DyP-type peroxidase (PmDyP), which generated phenol substituted aromatic compounds. Such enzymes PmDyP are molecularly cloned and expressed in a high growth strain followed by its purification and kinetic assays for further LCB biodegradation (**Yang et al., 2018**). Biosensor functional screening detected multicopper oxidase and CopA in *Pseudomonas* sp. and *P. stutzeri*, which respond to monomeric phenolic compounds (**Strachan et al., 2014**). GC-MS detected compounds such as 4-hydroxy-3methoxybenzoic acid and 2,6-dimethoxybenzen-1,4-diol were generated upon the action of CopA over lignin in the presence of Cu(II). In a study by **Granja-Travez and Bugg, (2018)**, CopA-type multicopper oxidase was elucidated from *P. fluorescens* Pf-5 and *P. putida* KT2440 CopA, which could oxidize 5,5′-dehydrodivanillate (DDVA) and guaiacylglycerol-β-guaiacyl ether (GGE) lignin compounds to form dimerized products (**Table 2**). Finally, the production of vanillic acid (VA) was done by the enzymatic action on lignosulfonate. The presence of *copA* gene was extremely important, as the mutation in *P. putida* KT2440 displayed a decrease in activity on aromatic compounds and this enzyme could act as laccase (**Granja-Travez and Bugg, 2018**). According to **Salvachúa et al. (2020),** the metabolism of lignin-based aromatic

compounds was mediated by outer membrane vesicles (OMVs), which served as an extracellular strategy in *P. putida* KT2440 for nutrient procurement.

5. Pseudomonas as cell factory: Advanced tools and technologies

P. putida KT2440 showed remarkable potential to synthesize and store PHAs by utilizing lignin and lignin model compounds as carbon sources (**Linger et al., 2014**). PHAs are biodegradable and biocompatible plastics that have a diverse range of applications in various fields such as packaging materials, drug carriers, and tissue implants (**Kumar et al., 2020a; 2016; Thakur et al., 2018**). *P. putida* KT2440 successfully utilized lignin and APL release after pretreatment of LCB as a carbon source for the biosynthesis of PHAs, which enabled the system to accumulate PHAs 32 wt.% in 48 h (**Linger et al., 2014**). Subsequently, ortho-cleavage of lignin derivative (catechol) forced *P. putida* to enhance the production of pyruvate and MA, which are raw materials for the fabrication of bioplastics (**Chio et al., 2019**). Even though the utilization of lignin for PHA production by *P. putida* KT2440 was demonstrated, more research and technological development are required to enhance the yield and quality of PHAs. **Salvachua et al. (2015**) used APL and screened out 14 oleaginous bacterial strains including *P. putida* that could accumulate PHAs as well as lipids. The bacterial lipids were further used for the production of biodiesel via the process of transesterification in the presence of acid or alkali catalyst (**Kumar et al., 2017a, b**).

Deployment of biotechnological tools in the field of bacterial PHAs production is demanding as it can modify the PHA biosynthesis pathway by insertion or deletion of genes (**Kumar et al., 2020a**). For example, **Wang et al.** (**2018**) reported the biosynthesis of mcl-PHA by *P. putida* A514 using lignin model compound (vanillate) as the only carbon source in the culture media. Improvement in the bacterial biomass and PHA accumulation was observed after targeted

overexpression of enzymes such as enoyl-CoA hydratase, long-chain fatty acid-CoA ligase and 3-hydroxyacyl-ACP thioesterase encoded by *phaJ4*, *alkK, and phaG* genes, respectively **Table 2**. The reported yield of 246 mg L^{-1} PHA signifies the relevance of advanced genetic tools and technologies in biovalorization of lignin by *pseudomonas* along with roles of various genes/enzymes involved in PHA biosynthesis pathways as illustrated by **Figure 1**.

Bacterial production of MA using lignin-based carbon sources is gaining attention. MA is the natural catabolic intermediate of lignin and lignin-based aromatics that is considered as a precursor molecule of AP and TA synthesis, which can be directly used as raw materials for the fabrication of marketable bio-based plastics (**Becker and Wittmann, 2019; Becker et al., 2018**; **Vardon et al., 2016**). Commercial-scale synthesis of unsaturated polyamides and unsaturated polyesters using MA as a precursor molecule was reported by **Suastegui et al.** (**2016**) and **Rorrer et al.** (**2017**). Bacterial MA production pathways using sugar as carbon sources have inherent limitations, resulting in low production yield and limiting the commercialization of this precursor molecule (**Becker and Wittmann, 2019**). Application of lignin and lignin-based aromatic as a carbon source for microbial production of MA remarkably changed the commercial production of cost-effective MA. There have been several wild and mutants/engineered microorganisms (**Table 1**) deployed recently for the production of MA at commercial scale (**Barton et al., 2018; Becker et al., 2018; Kohlstedt et al., 2018; Sonoki et al., 2018**). Owing to the fabulous performance of *P. putida* as aromatic compounds degrader and MA producer, many genetic engineering and manipulation approaches have been reported so far (**Lee et al., 2019; Xu et al., 2019b; Nikel and de Lorenzo, 2018**). The production of MA by chemically mutated *P. putida* KT2440 JD-1 strain using glucose-benzoate combination in the production media (**van Duuren et al., 2011**). Point mutation in the cat operon transcriptional regulator (*catR*) leads to

the suppression of the muconate cycloisomerase (*catB*) activities, resulting in biosynthesis of MA by JD-1 strain. Also, **van Duuren et al.** (**2012**) applied programmed pH-controlled glucose-benzoate feed and reported the MA yield 18.5 g L^{-1} with the production frequency of 0.6 g g^{-1} h^{-1}. These studies recapitulate how to enhance the yield of the anticipated products from lignin derivatives by altering the aromatic funneling pathways of the *P. putida* and regulating the physicochemical parameters of the growth media.

Upgrading MA production by metabolically engineered strain *P. putida* KT2440 has been performed by **Vardon et al. (2015**). An array of lignin derivatives or aromatics compounds such as, protocatechuate, coniferyl alcohol, phenol, ferulate, *p*-coumarate, vanillin, caffeate, 4-hydroxybenzoate (4HB), benzoate, and catechol have been chosen. The linking of natural protocatechuate degradation via β-ketoadipate pathway has been done by heterological *aroY* expression gene obtained from *E. cloacae*, which was able to translate enzyme protocatechuate decarboxylase. Concurrently, the disruption of natural protocatechuate metabolic pathway took place by enzyme protocatechuate 3,4-dioxygenase translated by *aroY* implemented on *pcaHG* locus. This strategy assisted *P. putida* to minimize the loss of aromatics in central metabolic pathway (**Vardon et al., 2015**). The utilization of phenol has been empowered by enzyme phenol monooxygenase translated by gene *dmpKLMNOP*, expressed in *Pseudomonas sp*. CF600 by metabolic engineering. Additionally, constitutively expression of *catA* gene encoded MA synthesizing enzyme catechol 1,2 dioxygenase and promoted the MA synthesis with minimal degradation. The replacement of genomic constituent such as *catR, catBC*, and the inherent promoter of *catBCA* of *P. putida* KT2440-CJ103 strain by strategically designed expression cassette was composed of the *tac* promotor (*Ptac*) along with *catA* and *dmpKLMNO*P genes. This manipulation led to the production of MA via the catechol and protocatechuate pathways

using diverse range of aromatic compounds. Within 78.5 h the bacterial strain yielded 13.5 g L^{-1} using glucose and aromatic (*p*-coumarate) as a growth and biotransformation material, respectively, in a fed-batch operation. Th purified MA was recovered (74%) with purity of more than 97% from bacterial culture by the use of activated carbon and subsequent crystallization (**Vardon et al., 2015**). Finally, the synthesis of bio-based AP was done by catalytic hydrogenation of produced MA using Pd/C-aided catalyst. **Johnson et al.** (**2016**) enhanced the MA productivity by improving the enzymatic activity (by supplying enzymatic flavin-derived cofactor) of protocatechuate decarboxylase involved in protocatechuate rout in *P. putida*. The reduction in protocatechuate accretion and 50% increment in MA production were observed using *p*-coumarate in the production media. The upstream aromatics degradation pathways of *P. putida* were reducing metabolic funneling of aromatics in the presence of aromatics mixture along with catechol as a key metabolic intermediate (**Nikel and de Lorenzo, 2018**). It was implemented to make engineered *E. coli* more competent towards the production of MA utilizing aromatics (**Thompson et al., 2018**).

Recently, **Kohlstedt et al.** (**2018**) advanced the metabolic ability of *P. putida* KT2440 and carried out the MA production using softwood lignin as feedstock. Adopting a subsequent chemical and biochemical transformation (hybrid) technology for MA production was demonstrated up to a pilot scale, which facilitated its application in the fabrication of bio-nylon. Firstly, thermal treatment was given to softwood lignin to produce a mixture of phenol and catechol along with minor quantities of p-cresol and o-cresol. Afterwards, genetically engineered MA-9 bacterial strain produced MA using the hydrolysate mixture within 54 h in the culture broth. The subsequent steps of purification, hydrogenation, and polycondensation with hexamethylenediamine allowed the production of bio-nylon (**Kohlstedt et al., 2018; Vardon et**

365 **al., 2015**). Also, the engineered MA-9 strain enabled the transformation of phenol to MA with
366 the help of native enzyme phenol hydroxylase, along with the production of methylated MA
367 from cresols. Using lignin hydrolysate as a carbon source, the MA-9 bacterial strain was able to
368 produce 13 g L^{-1} MA with minute quantities of methylated MA (**Kohlstedt et al., 2018**).
369 Bacterial funneling of lignin and lignin model compounds for the production of MA was innately
370 merged at catechol (toxic intermediate) node, which was the major stumbling block in
371 biovalorization of aromatic compounds. Metabolically engineered (deleted *catCB* gene) *P.*
372 *putida* KT2440-MA-1 was able to produce MA when a mixture of catechol-glucose was used in
373 the production media. However, the catechol noxiousness affected the metabolic energy level by
374 lowering the generation of ATP (adenosine triphosphate). The problem could be resolved by
375 adopting various approaches such as fermentation modes, control feeding, culture duration, etc.,
376 yielding a maximum production of 64.2 g L^{-1} MA, which was ten times higher than previously
377 reported values (**Kohlstedt et al., 2018**).

378 Improved *catA* gene expression, which translated catechol 1,2-dioxygenase enzyme
379 (catechol-degrader) in bacteria by system metabolic tools, has been considered as an emerging
380 strategy. Direct introduction of an additional set (*catA2*) of the gene in downstream of the *catA*,
381 whose expression was regulated by inherent *catA* promoter, was considered as the most effective
382 strategy among other biotechnological innovations (**Kohlstedt et al., 2018**). This strategy led to
383 the generation of a mutant MA-6, displayed enormous catechol tolerance, enhanced catechol
384 degradation, as well as improved the MA production with 98% purity at kilogram level. **Johnson**
385 **et al. (2017)** estimated the conversion efficiency of aromatics (*p*-coumarate and ferulate) by *P.*
386 *putida* KT2440 on a regulatory level. The lower biotransformation (*p*-coumarate and ferulate to
387 MA) efficiency was because of carbon catabolite repression (*crc*) present in genome of the

KT2440 strain. Deletion of *crc* regulator from genome of *P. putida* KT2440 significantly improved the MA yield (**Johnson et al., 2017**). However, the collective characteristic of the genus *Pseudomonas* in the above research studies was completely based on glucose as co-substrate (growth substrate) and lignin or lignin model compounds as production substrate (convertible substrate).

Recent investigations have been done to address the glucose-independent MA biosynthesis efficiency of genetically modified *Pseudomonas* strain and its production processes. **Salvachúa et al. (2018)** established the method and host selection to enhance the MA yield and the rate of production by utilizing lignin model compounds, namely hydroxycinnamic acid (HCA), p-coumaric acid (p-CA), and ferulic acid (FA). Recent development in bioprocess engineering and optimization of production process facilitated the production of MA by *P. putida* from aromatic compounds. Combined application of genetic and metabolic engineering resulted in the overexpression of desirable gene and deletion of a universal catabolic regulator, which ultimately led to the generation of robust strains. The production rate of MA was more than 0.5 g L^{-1} h^{-1} and the maximum yield was 50 g L^{-1}, which was close to the product tolerance (i.e., toxicity) limit of *P. putida*, in varying fermentation conditions (**Salvachúa et al., 2018**). Substrate feeding strategy at higher pH conditions significantly decreased the salt concentration, leading to higher product yield by reducing product dilution. Also, MA production was established with aromatics/lignin-rich feedstock (e.g., corn stover) and achieved 4 g L^{-1} yield (**Salvachúa et al., 2018**). **Sonoki et al.** (**2018**) constructed a lignin degrader *P. putida* IDPC strain by simultaneous removal of *pcaHG* and *catB* gene from the genome. Again, *pcaHG* expression was reinstated by the application of plasmid-based *pcaHG,* which was under control of the lac promoter, in that way escaping the typical expression regulator. Simultaneous biomass production and MA yield

411 was obtained by co-expression of the *pcd* gene encoding protocatechuate decarboxylase enzyme
412 obtained from *Klebsiella pneumoniae.* The molar yield of MA was 3%, 12% and 20% by using
413 vanillic acid (VA), 4-hydroxybenzoic acid (4-HB), and a mixture of both, respectively. The
414 lignin degrader *P. putida* IDPC construct was also reported for the production of MA from
415 softwood lignin hydrolysates, but this approach required additional research prior to large-scale
416 MA production (**Sonoki et al., 2018**).

417 **6. Research needs and future directions**

418 Biovalorization of widely available feedstock as feedstock is considered as beneficial for
419 both commercial as well as environmental point of view. Several worthwhile products such as
420 biofuel, chemicals, and biomaterials have been produced via lignin biovalorization by genus
421 *Pseudomonas* alone, as well as a hybrid system using advanced tools and technologies. The
422 generation of diverse range of lignin derived aromatic compounds can be achieved by
423 depolymerization of lignin. These depolymerized aromatics can be further converted into
424 valuable biological products via diverse microbial enzymatic action and funneling pathways. A
425 single bacterial system, every so often lacking the major vital enzymes, has a limited production
426 efficiency. Hence, the application of advanced genetic tools and metabolic engineering in genus
427 *Pseudomonas* can enhance the production efficiency of the system by hindering the formation of
428 unwanted (toxic) co-products.

429 The metabolic engineered bacterial strain *P. putida* has been enabled to use a wide range of
430 aromatic compounds for the biosynthesis of desirable products. Advancement in strategy of
431 lignin biovalorization built on the principle of sustainable environment and circular economy
432 will propel the integrated biorefinery towards sustainable alternatives. Additionally, this
433 approach may support the existing biorefinery technology economically. Even though a large

number of studies, demonstrations, and promising results have been published so far related to lignin biorefinery, lignin biorefinery is still underutilized at commercial level. Better engineering control of the bacterial system and its enzymatic interactions, genomic and metabolic engineering, and the screening and isolation of potential lignin-degraders (e.g., genus *Pseudomonas*) can make the lignin biorefinery more cost-effective and marketable. Life cycle assessment (LCA) of lignin valorization processes has demonstrated their reduced environmental impacts in comparison to the conventional fuel-based refinery. The biorefinery of lignin and its model compounds are gaining attention, and it is a key to ensure the best production route having the minimal environmental impacts. Until now, very few LCA investigations have been done for biovalorization of lignin. Existing studies mainly focus on the synthesis of phenolic chemicals and fuels (**Lettner et al., 2018; Bajwa et al., 2019**). Recently, a comparative LCA has been performed by **Corona et al. (2018)** for the production of AP from conventional fuel and lignin derivatives using hybrid system. They applied *P putida* KT2440 strain for the production of MA using lignin derivatives as feedstock. The lignin-derived AP displayed 4.87 kg CO_2 emission in the production of 1 kg AP, which was 78% less than mean and 62% less than maximum emission of GHGs from the fossil fuel-derived AP (**Corona et al., 2018**). Hence, the use of lignin-derivatives in biorefinery via a hybrid system can be more environmentally friendly than using lignin in the production of energy via direct combustion (i.e., the most common approach for lignin utilization). Further analysis is required to support and nurture the lignin biorefinery technology from infancy to its mature stage in the near future.

Conclusions

Biovalorization of lignin and lignin derivatives as feedstock for the production of fuels and chemicals can be a possible solution to reduce carbon footprint and encourage circular economy.

The robust enzymatic machinery and genetic modification of genus *Pseudomonas* make them a potential candidate for the upscaling of lignin-derived products such as PHAs, MA, AP, and pyruvate from the biomass-processing industries. Application of hybrid technologies, in which genus *Pseudomonas* plays a key role, can be a cost-effective and greener approach for future lignin biorefinery industry.

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