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## Bioconversion of a lignin-derived biphenyl dimer into the strategic building block 5-carboxyvanillic acid in *Pseudomonas putida* KT2440†

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The design of new biocatalysts for funneling lignin depolymerization-derived dimers into added-value compounds is nowadays a major challenge in biological lignin valorization. Biphenyl 5,5'-dehydrodivanillate (DDVA) is a model-lignin dimer that contains the C<sub>5</sub>–C<sub>5</sub>' linkage commonly found in lignin depolymerization mixtures. In this work, the metabolic potential of the industrially relevant *Pseudomonas putida* KT2440 bacterial strain was broadened by expressing synthetic DNA modules encoding selected metabolic and transport steps from the well-characterized DDVA degradation pathway of the *Sphingobium lignivorans* SYK-6 strain. By employing this heterologous expression strategy, we successfully developed an unprecedented resting cell-based bioprocess to convert DDVA into 5-carboxyvanillic acid (5CVA), a promising building block for the production of innovative bio-based polymers. This proof-of-concept study underscores the essential role of the associated DDVA transport systems. Furthermore, the findings reveal that *P. putida* KT2440 serves as an effective bacterial chassis for biotechnological processes that require the uptake of substrates through specific TonB-dependent transporters.

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### Green foundation

1. Sustainable management of lignin waste has predominantly focused on valorizing monomers, and limited studies have explored the conversion of lignin-derived dimers. Here, we present a green strategy for producing the key building block 5-carboxyvanillic acid (5CVA) using the biphenyl-type lignin dimer DDVA (biphenyl 5,5'-dehydrodivanillate) as the substrate.
2. The process was validated in the *Pseudomonas putida* KT2440 strain by expressing key metabolic steps of the DDVA degradation pathway from *Sphingobium lignivorans* SYK-6. DDVA uptake, a major bottleneck in the bioconversion, was addressed by cloning outer (DdvT) and inner (DdvK) membrane transporters, coupled with optimizing resting cell conditions, and a 5CVA yield of 30% was achieved.
3. Further research is required to: (i) optimize bacterial biocatalysts and the process to enhance 5CVA yields; (ii) implement the bioconversion using depolymerized lignin samples as feedstock; and (iii) scale up the bioprocess to meet industrial relevance.

## Introduction

In our societies, there is an urgent need for innovative waste management solutions that contribute to a transition towards a more sustainable circular economy. In particular, due to lignin recalcitrance, lignin recycling constitutes a challenge in the management of lignocellulosic biomass. After cellulose, lignin is considered the second most abundant plant-based biopolymer on Earth accounting for 30% of the organic carbon in the biosphere<sup>1</sup> and representing up to 40% of the energy accumulated in lignocellulosic biomass.<sup>2</sup> Lignin is extracted in

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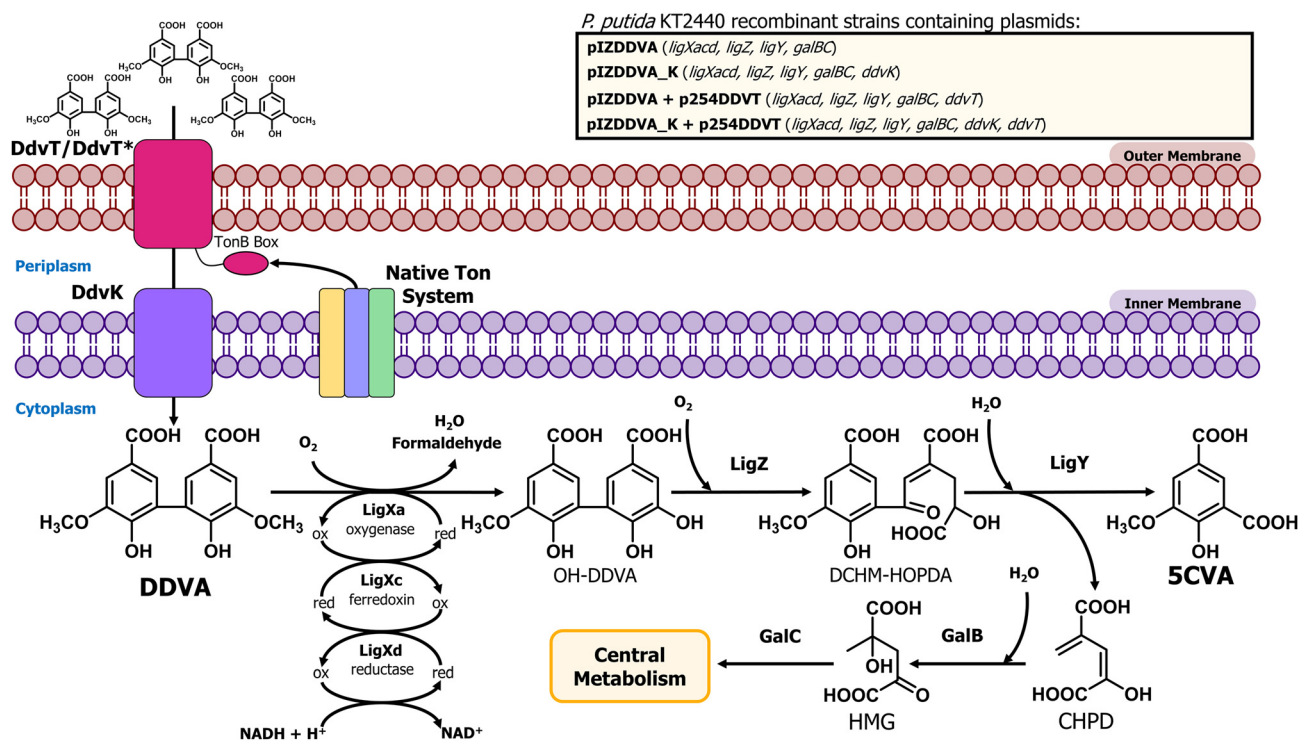
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amounts higher than 50 Mt per year<sup>3</sup> and is mostly considered a residue, usually burned for its fuel value.<sup>4</sup> However, as a massive reservoir of carbon and energy, lignin possesses a high potential value for being valorized through green biotechnology processes. In particular, from a circular economy perspective, the bio-conversion of lignin and its heterogeneous derived compounds to value-added products has emerged as an especially relevant strategy, and genetically modified bacteria have been used for the production of compounds of industrial relevance such as muconic acid,<sup>5,6</sup> adipic acid,<sup>7</sup> 2-pyrone-4,6-dicarboxylic acid,<sup>8–10</sup>  $\beta$ -keto adipic acid,<sup>11,12</sup> pyridine dicarboxylic acids,<sup>13–15</sup> substituted styrene molecules,<sup>16</sup> vanillin,<sup>17</sup> or polyhydroxyalkanoates,<sup>18–20</sup> among others.<sup>21</sup>

Chemo-catalytic lignin depolymerization methods mostly cleave carbon–oxygen linkages such as  $\beta$ -O-4 aryl ether bonds, thus enriching lignin streams in dimers and oligomers with carbon–carbon linkages.<sup>22</sup> One of the most prevalent carbon–carbon linkages between benzene rings in lignin is the C<sub>5</sub>–C<sub>5'</sub> linkage found in biphenyl dimers.<sup>23–25</sup> Kraft lignin contains additional biphenyl units caused by radical coupling reactions.<sup>26</sup> Therefore, designing biological systems capable of bio-converting recalcitrant lignin-derived biphenyls into value-added products could significantly enhance lignin waste man-

agement. This approach would expand current metabolic funneling processes, which are primarily focused on valorizing monomers derived from the cleavage of carbon–oxygen linkages. In particular, 5,5'-dehydrodivanillate (DDVA) has been used as a model of lignin-derived biphenyl compounds and it can be obtained as a result of different lignin depolymerization methods. Thus, this chemical can be generated *via* fungal depolymerization processes, as in the case of wood decay by *Phanerochaete chrysosporium*,<sup>27,28</sup> as well as by using physico-chemical procedures like the alkaline nitrobenzene oxidation of lignin samples<sup>29</sup> or as an oxidation product of lignosulfonates.<sup>30</sup> Alternatively, DDVA can be obtained through enzymatic oligomerization of vanillic acid, another lignin-derived product, using fungal laccases.<sup>31</sup> The DDVA degradation pathway has been well characterized in the bacterium *Sphingobium lignivorans* SYK-6.<sup>32</sup> The first step of DDVA catabolism includes an *O*-demethylation carried out by a multicomponent oxygenase composed of LigXa (oxygenase), LigXc (ferredoxin) and LigXd (ferredoxin reductase) subunits, generating 2,2',3-trihydroxy-3'-methoxy-5,5'-dicarboxybiphenyl (OH-DDVA) (Fig. 1).<sup>33,34</sup> In the second step, the extradiol dioxygenase LigZ is able to cleave one of the aromatic rings generating a *meta*-ring cleavage compound (4,11-dicarboxy-8-hydroxy-9-methoxy-



**Fig. 1** Scheme of transport and catabolic steps for 5CVA production from the lignin-derived DDVA dimer in recombinant *P. putida* KT2440 strains. General scheme of the metabolic capabilities implemented in *P. putida* KT2440. The metabolic steps needed to convert DDVA into 5CVA (LigXacd, LigZ, and LigY from *S. lignivorans* SYK6) and those needed for the degradation of CHPD (GalB and GalC from *P. putida* KT2440) are encoded in plasmid pIZDDVA. The DdvK inner membrane DDVA transporter from strain SYK6 was added to pIZDDVA yielding plasmid pIZDDVA\_K. Plasmid p254DDVT encodes the SYK6 outer membrane DDVA transporter DdvT. Plasmid p254DDVTB encodes a chimeric DdvT\* protein with a synthetic TonB box. Abbreviations: DDVA, 5,5'-dehydrodivanillate; OH-DDVA, 2,2',3-trihydroxy-3'-methoxy-5,5'-dicarboxybiphenyl; DCHM-HOPDA, 4,11-dicarboxy-8-hydroxy-9-methoxy-2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate; CHPD, 4-carboxy-2-hydroxypenta-2,4-dienoate; HMG, 4-hydroxy-4-methyl-2-oxoglutarate; and 5CVA, 5-carboxyvanillic acid.



2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate) (DCHM-HOPDA) that is subsequently hydrolyzed by the LigY hydrolase to yield 5-carboxyvanillate (5CVA) and 4-carboxy-2-hydroxypenta-2,4-dienoate (CHPD)<sup>35,36</sup> (Fig. 1). 5CVA is converted to vanillate by the LigW and LigW2 decarboxylases<sup>37,38</sup> and then redirected to the central metabolism through the protocatechuate 4,5-cleavage pathway.<sup>39</sup>

Interestingly, 5CVA, an intermediate in the DDVA pathway, is a structural analogue of isophthalic acid but with enhanced oxygen functionalities. This makes it a highly promising building block for the development of innovative bioplastics. In fact, using artificial intelligence-based approaches, it has been recently demonstrated that 5CVA presents optimal characteristics as a novel building block for the production of bio-based polymers. Notably, this diacid can be polymerized into poly(ethylene 5-carboxyvanillate) (PEC), a polyester that exhibits superior properties compared to polyethylene terephthalate (PET).<sup>40</sup> With the growing concern over the environmental impact of petrochemical-based plastics,<sup>41</sup> the development of bioplastics from renewable feedstocks offers a crucial and sustainable alternative. In support of this goal, our study introduces a novel biotechnological approach for the bioconversion of a lignin-derived aromatic dimer, DDVA, into the valuable 5CVA building block.

*P. putida* KT2440 is a non-pathogenic industrially relevant bacterium that has been extensively used for lignin valorization.<sup>6,10,12,15,20,21,42–44</sup> In this work, a genetically engineered *P. putida* KT2440 strain was developed to express a specific set of genes from *S. lignivorans* SYK6, enabling the bioconversion of DDVA into 5CVA. A successful growth-independent biotransformation, known as a resting cell process, was established as a novel procedure for the production of 5CVA.

## Experimental

### Chemicals, bacterial strains, plasmids and growth conditions

All chemicals were purchased from Merck unless otherwise stated. DDVA and 5CVA were purchased from Biosynth and APIN Chemicals Ltd, respectively. DDVA and 5CVA 0.05 M stock solutions were prepared in water after neutralization with equimolar amounts of NaOH and stored at  $-20\text{ }^{\circ}\text{C}$ . The bacterial strains and plasmids used in this work are detailed in Table 1. *P. putida* KT2440 strains and *S. lignivorans* SYK-6 were cultivated in liquid lysogeny broth (LB) medium<sup>45</sup> at  $30\text{ }^{\circ}\text{C}$  and 200 rpm agitation. M63 minimal medium was also used to grow *P. putida* KT2440 strains.<sup>46</sup> *Escherichia coli* strains were cultivated in LB medium at  $37\text{ }^{\circ}\text{C}$  and 200 rpm agitation. When required, gentamicin ( $10\text{ }\mu\text{g ml}^{-1}$ ) or kanamycin ( $50\text{ }\mu\text{g ml}^{-1}$ ) was added to the growth medium.

### Molecular biology techniques

Standard molecular biology techniques were performed as previously described.<sup>45</sup> Plasmid DNA extractions were performed using a High Pure Plasmid Isolation Kit (Roche Applied Science). DNA fragments were purified from agarose gels or

Table 1 Bacterial strains and plasmids used in this study

	Relevant genotype	Reference or source
<b>Bacterial strains</b>		
<i>E. coli</i> DH10B	<i>F<sup>-</sup>, mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZAM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG</i>	Invitrogen
<i>P. putida</i> KT2440	Wild-type strain	Bagdasarian <i>et al.</i> <sup>47</sup>
<i>S. lignivorans</i> SYK-6	Wild-type strain	Masai <i>et al.</i> <sup>32</sup>
<b>Plasmids</b>		
pIZ1016	Gm <sup>R</sup> , broad-host range expression vector bearing <i>lacF<sup>l</sup></i> and <i>P<sub>lac</sub></i> , and the pBBR1MCS replication origin	Moreno-Ruiz <i>et al.</i> <sup>48</sup>
pSEVA254	Km <sup>R</sup> , broad-host range expression vector bearing <i>lacF<sup>l</sup></i> and <i>P<sub>lac</sub></i> , and the RSF1010 replication origin	Silva-Rocha <i>et al.</i> <sup>49</sup>
pSEVA234	Km <sup>R</sup> , broad-host range expression vector bearing <i>lacF<sup>l</sup></i> and <i>P<sub>lac</sub></i> , and the pBBR1 replication origin	Silva-Rocha <i>et al.</i> <sup>49</sup>
pSEVA238	Km <sup>R</sup> , broad-host range expression vector bearing <i>xylS</i> and <i>P<sub>lac</sub></i> , and the pBBR1 replication origin	Silva-Rocha <i>et al.</i> <sup>49</sup>
pIZDDVA	Gm <sup>R</sup> , pIZ1016 derivative expressing <i>ligXaXcXd</i> , <i>ligZ</i> , <i>ligY</i> , <i>galBC</i> under the <i>lacF<sup>l</sup>/P<sub>lac</sub></i> regulatory couple.	This work
pIZDDVA_K	Gm <sup>R</sup> , pIZ1016 derivative expressing <i>ligXaXcXd</i> , <i>ligZ</i> , <i>ligY</i> , <i>galBC</i> under the <i>lacF<sup>l</sup>/P<sub>lac</sub></i> regulatory couple	This work
p254DDVT	Km <sup>R</sup> , pSEVA254 derivative expressing <i>dhvT</i> under the <i>lacF<sup>l</sup>/P<sub>lac</sub></i> regulatory couple	This work
p254DDVTB	Km <sup>R</sup> , pSEVA254 derivative expressing a chimeric <i>dhvT*</i> gene with the consensus KT2440 TonB box "LELPAVITA" under the <i>lacF<sup>l</sup>/P<sub>lac</sub></i> regulatory couple	This work



PCR mixtures with a Gene Extraction Kit or a PCR Purification Kit (Qiagen), respectively. Gibson Assembly reactions were performed using an NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs). The oligonucleotides employed (see Table S1 of the ESI†) were supplied by IDT. All cloned inserts and DNA fragments were confirmed by DNA sequencing in an ABI Prism 377 automated DNA sequencer. Transformation of *E. coli* cells was carried out by using the RbCl method.<sup>45</sup> Plasmids were transferred to *P. putida* KT2440 cells by electroporation (Gene Pulser, Bio-Rad).<sup>45</sup>

### Construction of plasmids pIZDDVA, pIZDDVA\_K, p254DDVT, and p254DDVTB

**Construction of pIZDDVA\_K.** *ligXa*, *ligY*, *ligZ*, *ligXc*, and *ligXd* genes were amplified from *S. lignivorans* SYK-6 genomic DNA maintaining their original codon usage by using the primer pairs 5ligX/3ligX, 5ligY/3ligY, 5ligZ/3ligZ, *ligXc\_Fw/ligXc\_Rv*, and *ligXd\_Fw/ligXd\_Rv*, respectively (Table S1†). *ligXa*, *ligZ*, and *ligY* PCR products were cloned into pSEVA238 by HiFi DNA Assembly. The *ligXc* and *ligXd* PCR products were subsequently cloned into the previous construction by *XbaI/SpeI* double digestion and ligation. For correction in the *ligZ* intergenic region, *ligXa*, *ligZ* and *ligY* genes were again amplified by PCR from the pSEVA238\_ligXaZYXcXd plasmid using the primer pairs *ligXa\_Fw\_KpnI/ligXa\_Rv\_NheI\_NdeI*, *ligZ\_Fw\_NdeI/ligZ\_Rv\_XhoI*, and *ligY\_Fw\_NheI\_XhoI/ligY\_Rv\_BamHI*, respectively. These PCR fragments were cloned in pSEVA238\_ligXaZYXcXd substituting the original *KpnI-BamHI* fragment. The *galBC* and *ddvK* genes were PCR-amplified by using the primer pairs *galBC\_Fw\_AvrII\_EcoRI/galBC\_Rv\_SacI* and *MFS\_Fw\_AvrII/MFS\_Rv\_EcoRI* and by using as template *P. putida* KT2440 or *S. lignivorans* SYK-6 genomic DNAs, respectively. These two PCR fragments were cloned in a pSEVA234 vector double-digested with *AvrII* and *SacI*. Finally, the *KpnI-SpeI* fragment from pSEVA238\_ligXaZYXcXd containing the *lig* genes was cloned in the *KpnI/SpeI* double-digested pSEVA234\_ galBCddvK yielding plasmid pSEVA234\_galBC\_ddvK\_ ligXaZYXcXd. Due to plasmid instability reasons, the *galBC\_ddvK\_ ligXaZYXcXd* DNA fragment was PCR-amplified using the primer pair *XmaI\_lig frag\_Fw/SpeI\_lig frag\_Rv*, and it was cloned into the *XmaI/SpeI*-double digested pIZ1016 vector yielding plasmid pIZDDVA\_K (Fig. S1†).

**Construction of pIZDDVA.** Plasmid pIZDDVA\_K was amplified using primers oCDCS7 and oCDCS8 (Table S1†). This PCR product was treated with the *DpnI* restriction enzyme and religated using Gibson Assembly, yielding plasmid pIZDDVA (Fig. S1†).

**Construction of p254DDVT.** *ddvT* was amplified from *S. lignivorans* SYK-6 genomic DNA using the primer pair *ddvT\_Fw\_SD\_XbaI/ddvT\_Rv\_NheI* (Table S1†), and the PCR product was cloned into the *XbaI* site of the pSEVA254 vector, generating plasmid p254DDVT (Fig. S1†).

**Construction of p254DDVTB.** The 5' and 3' regions of the *ddvT* gene were PCR-amplified from p254DDVT using primer pairs *ddvT\_Fw\_SD\_XbaI/LELPATVITA\_Rv* and *LELPATVITA\_Fw/*

*ddvT\_Rv\_HindIII*, respectively (Table S1†). The two obtained fragments were then used as templates for SOE (splicing overlap extension)-PCR using primers *ddvT\_Fw\_SD\_XbaI* and *ddvT\_Rv\_HindIII*. The 2924 bp-DNA product was cloned into the *XbaI/HindIII* double-digested pSEVA254 plasmid, yielding plasmid p254DDVTB.

The original signal peptide of the DdvT protein has been maintained for its heterologous expression in *P. putida* KT2440.

### DDVA bioconversion in *P. putida* resting cell assays

1 mL of an overnight seed culture of the different *P. putida* KT2440 recombinant strains, containing different combinations of plasmids pIZ1016, pSEVA254, pIZDDVA, pIZDDVA\_K, p254DDVT or p254DDVTB and grown in LB with kanamycin and gentamycin, was used to inoculate 200 mL of fresh LB medium without antibiotics. They were incubated for 3 h at 30 °C. 1 mM IPTG was then added to induce expression of *galBC*, *ligXacdYZ*, *ddvT*, and *ddvK* genes, followed by an additional 24 h incubation period. Cells (equivalent to an OD<sub>600</sub> of 70 or to 0.28 ± 0.05 g dry cell weight) were collected at 8500 rpm for 15 minutes at 15 °C and then resuspended in 10 mL of the resting cell medium, unless otherwise stated: 50 mM sodium phosphate buffer pH 7.5 supplemented with the aromatic substrate (1 mM DDVA) and 0.1% glucose. For biotransformations performed with cells grown in M63 mineral medium, this was supplemented with 1 mM MgSO<sub>4</sub>, trace elements and 0.034 mM EDTA as previously described<sup>50</sup> and either 20 mM glucose or 15 mM sodium octanoate (carbon/nitrogen ratio equal to 4 (mol/mol) in both cases) as carbon sources. When required, resting cell experiments were also performed in sodium phosphate buffer pH 6 or with higher amounts of the substrate (5 mM DDVA). These resting cell mixtures were then incubated at 30 °C and 200 rpm agitation in a 50 mL flask. A 1 mL sample was taken at different time points, filtered through 0.45 µm pore size filters and stored at -20 °C for further analysis of metabolites accumulated in the supernatant.

### Metabolite analyses

To monitor 5CVA production from DDVA, both DDVA and 5CVA stock solutions and the supernatant of the samples collected were diluted in 0.1 M sodium phosphate buffer pH 6.5 and analyzed by HPLC-MS (high-performance liquid chromatography coupled to mass spectroscopy) using 1260 Infinity II and Infinity Lab LC/MSD XT systems (both from Agilent) and a Poroshell 120 EC-C18 (4.6 × 100 mm, 4 µm, Agilent) reverse phase column. DDVA and 5CVA were detected and quantified at 230 nm in a diode array type of UV/VIS detector (DAD). The solvents used for HPLC analyses were water/0.1% formic acid (solvent A) and acetonitrile/0.1% formic acid (solvent B). The applied gradient for analysis was 0–40% of solvent B for 12 min followed by a 3 min column re-equilibration step in which solvent B is restored to 0%. The flow rate was always kept at 1 mL min<sup>-1</sup>. Chromatograms generated from the samples were compared to commercial standards. The identity of DDVA and 5CVA was confirmed by extracted ion analysis for fragments 334 and 212 *m/z*, respectively, in positive ion mode,



and they were compared with the same standard-generated profiles.

### *In silico* identification of a TonB box

*P. putida* KT2440 TonB-dependent transporters annotated by Cornelis & Bodilis<sup>51</sup> were considered for TonB box detection. The first 50 amino acids of all 27 receptors were aligned using MUSCLE<sup>52</sup> excluding signal peptides. Alignments of the putative TonB region were corrected manually using the corresponding sequence of the characterized BtuB receptor of *P. aeruginosa*.<sup>53</sup> 11 *P. putida* KT2440 putative TonB box sequences were selected due to their similarity to DdvT and BtuB sequences and the resulting consensus sequence was obtained.

## Results and discussion

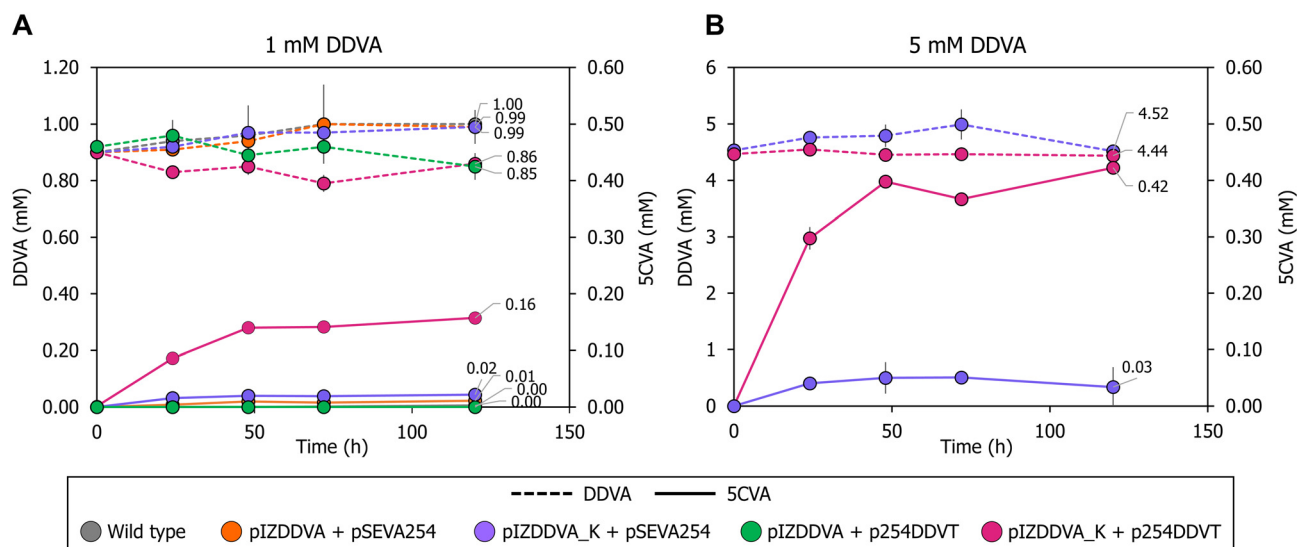
### Construction of a *P. putida* KT2440 recombinant biocatalyst for the bioconversion of DDVA into 5CVA

As indicated in the Introduction, *S. lignivorans* SYK-6 can degrade DDVA with the formation of 5CVA, but then this intermediate is further channeled to the central metabolism of the cell and it does not accumulate as the final product.<sup>54</sup> Thus, to develop a bacterial biocatalyst able to accumulate 5CVA from DDVA, we have engineered the wild-type *P. putida* KT2440 strain, which is unable to degrade or modify 5CVA, by expressing the SYK-6 genes required to convert DDVA into 5CVA. To accomplish this, *P. putida* KT2440 was transformed with plasmid pIZDDVA that contains the *ligXa*, *ligXc*, *ligXd*, *ligZ*, and

*ligY* genes from strain SYK-6 (Fig. 1) under control of the *lacI<sup>d</sup>*/*P<sub>tac</sub>* regulatory couple, allowing gene induction when required by the addition of IPTG. During DDVA metabolism, the activity of the LigX, LigZ and LigY enzymes produces not only 5CVA but also 4-carboxy-2-hydroxypenta-2,4-dienoate (CHPD) (Fig. 1),<sup>35</sup> a molecule that has been proposed to be metabolized to pyruvate by the GalB hydratase and the GalC aldolase, two enzymes of the gallate degradation pathway in *P. putida* KT2440 and that are functionally equivalent to LigJ and LigK from strain SYK6, respectively.<sup>55,56</sup> Therefore, the *galB* and *galC* genes were also cloned into pIZDDVA (Fig. 1), to promote CHPD consumption and avoid a potential product inhibition of LigY, caused by the accumulation of CHPD, hence optimizing 5CVA production.

When the recombinant *P. putida* KT2440 (pIZDDVA) strain was tested, we did not observe growth in minimal medium containing 5 mM DDVA as the sole carbon source. Moreover, when resting cells of the same strain were assayed in the presence of 1 mM DDVA, only a very minor amount (0.01 mM) of 5CVA was detected after 120 h of incubation (Fig. 2). In the wild-type KT2440 strain, the 5CVA production was completely undetectable at any time (Fig. 2). These results suggested that the heterologous expression of the genetic determinants that encode the enzymes needed for the conversion of DDVA into 5CVA was not enough to allow 5CVA production in the recombinant *P. putida* cells.

It is known that the Major Facilitator Superfamily (MFS) inner membrane transporter DdvK and the Ton system-dependent outer membrane transporter DdvT are required for efficient uptake and catabolism of DDVA in *S. lignivorans*



**Fig. 2** 5CVA production profiles from DDVA by several *P. putida* KT2440 recombinant strains. Production of 5CVA (mM) using different *P. putida* KT2440 strains in resting cell assays with 1 mM (A) or 5 mM (B) DDVA as the substrate. DDVA consumption and 5CVA production are indicated with discontinuous and continuous lines, respectively. The strains tested were wild-type *P. putida* KT2440 containing control plasmids pI21016 and pSEVA254 (grey); KT2440 containing plasmid pIZDDVA and the control plasmid pSEVA254 (orange); KT2440 containing plasmid pIZDDVA\_K and the control plasmid pSEVA254 (purple); KT2440 containing plasmid pIZDDVA and plasmid p254DDVT (green); and KT2440 containing plasmid pIZDDVA\_K and plasmid p254DDVT (pink). Samples were taken at 0, 24, 48, 72 and 120 h. All the resting cell experiments were performed in triplicate. Error bars represent standard deviation of the triplicates.



SYK-6.<sup>57–59</sup> Unlike DdvK, which seems completely essential for DDVA uptake independently of the compound concentration in the culture medium, DdvT appears to be partially replaceable by unknown outer membrane porins or transporters in the presence of higher amounts (1 to 5 mM) of DDVA.<sup>57,58</sup> Since neither the DdvK nor the DdvT ortholog is present in *P. putida* KT2440, we checked the contribution of these two DDVA transport elements in the previously engineered *P. putida* KT2440 strain expressing the *ligXYZgalBC* genes. For this purpose, *ddvK* was engineered *in cis* with the *ligXYZgalBC* genes yielding plasmid pIZDDVA\_K (Fig. 1). For *ddvT* expression under control of the *lacI<sup>q</sup>/P<sub>trc</sub>* regulatory couple, plasmid p254DDVT was constructed (Fig. 1). As shown in Fig. 2A, *ddvK* co-expression with the catabolic genes turned into an earlier 5CVA generation in resting cells, although the biotransformation efficiency was still low (production of 0.02 mM 5CVA after 48 hours). In contrast, the expression of the catabolic genes and *ddvT* did not allow the detection of 5CVA (Fig. 2A). However, the simultaneous expression of *ddvK*, *ddvT*, and the catabolic genes in the *P. putida* KT2440 (pIZDDVA\_K, p254DDVT) strain boosted DDVA utilization, reaching up to 0.16 mM 5CVA production after 48 h of resting cell assay (Fig. 2A). Therefore, these results show for the first time that bacterial biocatalysts can be successfully engineered for the bioconversion of DDVA into 5CVA.

The production of 5CVA by *P. putida* KT2440 (pIZDDVA\_K, p254DDVT) resting cells increased to 0.42 mM when 5 mM DDVA was used as the substrate (Fig. 2B). This suggests that the enzymatic machinery responsible for converting DDVA into 5CVA is not yet saturated at a substrate concentration of 1 mM DDVA. The relatively low production yield of 5CVA (approximately 16% in the presence of 1 mM DDVA), which results in a significant amount of DDVA substrate remaining in the culture medium, combined with the absence of detectable metabolic intermediates from DDVA degradation other than 5CVA (Fig. S2†), strongly suggests that the primary limitation in using *P. putida* as a biocatalyst for 5CVA production is the uptake of DDVA into the bacterial cells, rather than the enzymatic activity responsible for its degradation.

#### DdvT is recognized by the native Ton system of *P. putida* KT2440

The results presented above demonstrate that active DDVA uptake through the outer membrane of *P. putida* is crucial for its subsequent bioconversion to 5CVA. This implies that the native inner membrane Ton system in *P. putida* KT2440 can, at least to some extent, interact with the outer membrane DdvT transporter from *S. lignivorans* SYK-6. However, since DDVA transport efficiency appears to be a significant bottleneck in the bioconversion of DDVA to 5CVA, we hypothesized that DdvT may not operate optimally with the native Ton machinery of KT2440. The genome of KT2440 contains annotations for at least one Ton system (PP\_5306, PP\_5307, and PP\_5308) and several TonB-like genes (PP\_0696, PP\_1221, PP\_1512, and PP\_4994). Consequently, we sought to optimize this transport step. Initially, we attempted to clone the DdvT-associated Ton system,

which has been identified as essential for SYK-6 survival and DDVA uptake.<sup>58</sup> The *tonB1-exbB1-exbD1-exbD2* genes were PCR-amplified from the SYK-6 genome and they were tried to be cloned into the p254DDVT plasmid under the control of the *lacI<sup>q</sup>/P<sub>trc</sub>* regulatory system. However, no stable plasmid constructions were obtained, suggesting that the expression of these membrane proteins together with DdvT might disrupt cellular functionality in *E. coli* when using a pSEVA254 vector. Alternatively, a second approach involved replacing the reported TonB box of DdvT with a synthetic TonB box designed to be more compatible with the native inner membrane Ton system of KT2440. To achieve this, the DdvT TonB box motif (I-I-V-T)<sup>58</sup> was aligned with the corresponding region of 27 different TonB-dependent transporters annotated in KT2440.<sup>51</sup> From this alignment, a TonB box containing the L-X-X-X-V/I-T motif was identified in 11 of these outer membrane transporters, as well as in the well-characterized BtuB receptor of *P. aeruginosa*<sup>53</sup> (Fig. S3A†). Notably, the alignment revealed a fully conserved leucine residue that was absent in the TonB box of DdvT from strain SYK-6 (Fig. S3A†). To determine whether this leucine was crucial for establishing an efficient interaction between DdvT and the native Ton system of KT2440, a synthetic consensus TonB box (L-E-L-P-A-T-V-I-T-A amino acid sequence) was introduced to replace the corresponding residues in DdvT, generating a DdvT\* chimeric protein in plasmid p254DDVTB (Fig. 1). *P. putida* KT2440 (pIZDDVA\_K, p254DDVTB) resting cell assays revealed that the chimeric DdvT\* transporter was functional and DDVA was converted to 5CVA (Fig. S3B†). However, the 5CVA production yield using the DdvT\* chimeric transporter was not higher than that obtained with the wild-type DdvT protein (Fig. 2A). This finding suggests that the bottleneck in DDVA uptake is unlikely to result from a deficient interaction between the SYK-6 DdvT transporter and the KT2440 inner membrane Ton machinery. Furthermore, these results demonstrate that *P. putida* KT2440 can serve as a versatile microbial platform for the functional expression of heterologous TonB-dependent transporters, even those with specificity closely aligned to their native inner membrane Ton machinery.

#### Enhancing 5CVA production

The initial experiments demonstrated a low yield of 5CVA. To assess whether the culture medium used for biocatalyst growth affects 5CVA production efficiency during the resting cell process, we compared the results obtained when cultivating *P. putida* KT2440 (pIZDDVA\_K, p254DDVT) cells in LB medium and minimal media containing two different carbon sources, *i.e.*, glucose and octanoate, leading to biocatalysts with varying energy and reducing power levels.<sup>60</sup> The efficiency of 5CVA production was similar when using cells grown in LB medium or glucose-containing minimal medium. However, cells grown in octanoate-containing minimal medium appear to lead to a lower production yield (Fig. S4†). These findings indicate that the choice of carbon source during cell cultivation is crucial in determining the 5CVA production yield, with LB medium being a suitable choice for growing the *P. putida* KT2440 (pIZDDVA\_K, p254DDVT) biocatalyst.



We then investigated whether modifications in the resting cell conditions could enhance 5CVA production. The results previously presented indicate that the outer membrane DdvT transporter is functional in *P. putida* KT2440, and hence it is likely that the transport bottleneck limiting DDVA bioconversion to 5CVA is due to the inner membrane Ton system and/or to the DdvK MFS transporter.<sup>57</sup> MFS transporters responsible for the uptake of lignin-derived aromatic compounds belong to either the aromatic acid/H<sup>+</sup> symporter (AAHS) or the metabolite/H<sup>+</sup> symporter (MHS) families, which rely on a proton gradient across the inner membrane.<sup>61</sup> Similarly, the proton motive force also powers the activity of TonB-dependent transporters.<sup>61</sup> To enhance DDVA transport across the membrane, we conducted resting cell assays at lower pH levels to increase the proton concentration in the periplasm,<sup>62</sup> thereby boosting the proton motive force required for the TonB-dependent DdvT activity and for the DdvK activity.<sup>63–65</sup> At pH 6, *P. putida* KT2440 (pIZDDVA\_K, p254DDVT) resting cells achieved a 5CVA production of 0.3 mM from 1 mM DDVA substrate (Fig. 3), representing a 30% yield, higher than the 16% yield observed at pH 7.5 (Fig. 2A). Notably, no increase in 5CVA production was observed at pH 6 when using *P. putida* KT2440 (pIZDDVA\_K, pSEVA254) resting cells (Fig. 3), reinforcing the notion that passive diffusion of non-charged (protonated) DDVA across cell membranes is an inefficient transport system and that both DdvK and DdvT are crucial for effective active DDVA uptake. In summary, lowering the pH in resting cell assays proved to be an effective strategy to overcome DDVA transport limitations, leading to a substantial improvement in 5CVA production yields.

Although lowering the pH during resting cell incubation improved the 5CVA production yield, further optimization of the

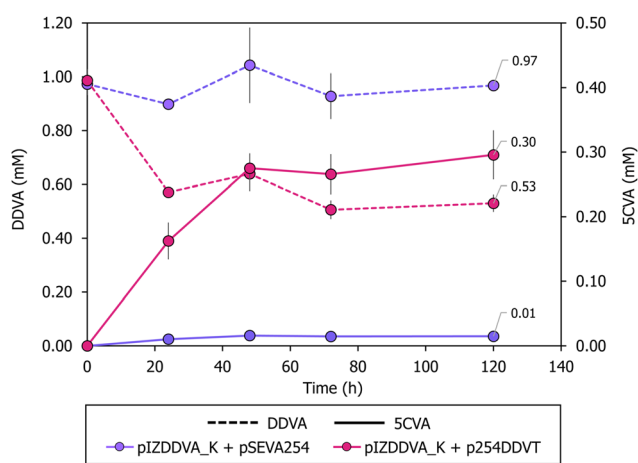
DDVA bioconversion process would be necessary to achieve higher 5CVA production levels. DDVA transport system may still be underperforming; and therefore, further optimization is advisable. For instance, optimizing the signal peptide of DdvT to ensure proper recognition by the *P. putida* secretion machinery could enhance transport efficiency.<sup>66</sup> On the other hand, cellular toxicity of certain DDVA pathway products, e.g., formaldehyde, and intermediates, e.g., DCHM-HOPDA (Fig. 1),<sup>35</sup> should be mitigated. In this sense, we observed that DDVA bioconversion was undetectable without glucose supplementation in the resting cell medium. This observation aligns with previous studies demonstrating that adding glucose increases intracellular NAD(P)H levels, which helps counteract the toxicity of compounds generated during the *O*-demethylation of aromatic substances.<sup>10,67,68</sup> Enhancing DDVA bioconversion efficiency can also be achieved by optimizing enzyme dosage and functionality. Strategies such as codon-usage optimization, employing alternative transcription and translation signals, and rational enzyme dosing (an optimal ratio of 1 : 30 : 2 has been proposed for LigXa : LigXc : LigXd),<sup>33</sup> are promising. To mitigate potential metabolic burdens associated with the DDVA pathway in recombinant *P. putida* biocatalysts, chromosomal integration of the synthetic gene cassette followed by adaptive laboratory evolution (ALE) could also be beneficial. Finally, the reuse of the optimized recombinant biocatalyst could further increase the 5CVA production levels.

## Conclusions

Previous studies have demonstrated that recombinant strains of *P. putida* KT2440 are effective hosts for the valorization of various lignin-derived monoaromatics.<sup>6,10,42–44</sup> More recently, it has been shown that a recombinant *P. putida* KT2440 strain expressing the LsdE and LsdA enzymes from *Novosphingobium aromaticivorans* DSM12444 acquired the ability to catabolize the dimer *erythro*-DGPD (1,2-diguaiacylpropane-1,3-diol), and this pathway has been further engineered to produce *cis,cis*-muconic acid.<sup>69</sup>

This study broadens the current understanding of *P. putida* KT2440 as a versatile bacterial chassis for lignin valorization by demonstrating that the heterologous expression of the *ligXacdZY* genes from *S. lignivorans* SYK6 enables the successful bioconversion of the biphenyl-type lignin dimer DDVA into the strategic building block 5CVA. Unlike *erythro*-DGPD, which does not appear to require a specific transport system in *P. putida*, DDVA uptake depends on a dedicated active transport system involving an outer membrane TonB-dependent transporter (DdvT) and an inner membrane MFS transporter (DdvK). This transport system has been identified as a major bottleneck in the conversion of DDVA to 5CVA, but optimizing resting cell conditions—such as lowering the pH—can help overcome this limitation.

Overall, this work highlights the importance of engineering not only metabolic pathways but also transport systems for the efficient valorization of certain lignin-derived compounds in



**Fig. 3** 5CVA production profiles from DDVA in *P. putida* resting cells at pH 6. Production of 5CVA (mM) using different *P. putida* KT2440 strains in resting cell assays performed at pH 6 with 1 mM DDVA as the substrate. DDVA consumption and 5CVA production are indicated with discontinuous and continuous lines, respectively. The strains tested were *P. putida* KT2440 containing plasmid pIZDDVA\_K and the control plasmid pSEVA254 (purple), and *P. putida* KT2440 containing plasmid pIZDDVA\_K and plasmid p254DDVT (pink). Samples were taken at 0, 24, 48, 72 and 120 h. All the resting cell experiments were performed in triplicate. Error bars represent standard deviation of the triplicates.



heterologous hosts. Additionally, it reveals for the first time that *P. putida* KT2440 is a promising bacterial chassis for biotechnological processes requiring the uptake of substrates via a specific TonB-dependent transporter. This proof-of-concept study also lays the foundation for developing a sustainable strategy to produce 5CVA, a strategic building block for the synthesis of novel bio-based polymers with enhanced properties.<sup>40</sup>

## Author contributions

Helena Gómez-Álvarez: conceptualization, investigation, formal analysis, writing – original draft, and writing – review & editing; Carlos del Cerro-Sánchez: conceptualization, investigation, formal analysis, writing – original draft, writing – review & editing, and funding acquisition; Pablo Iturbe: investigation; Virginia Rivero-Buceta: methodology; Juan Nogales: conceptualization and writing – review & editing; Timothy D. H. Bugg: conceptualization, writing – review & editing, and funding acquisition; and Eduardo Díaz: conceptualization, writing – original draft, writing – review & editing, and funding acquisition.

## Data availability

All the generated data are available within this manuscript and its ESI.†

## Conflicts of interest

The authors declare no conflict of interest.

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