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Bioconversion of lignin-derived aromatics into the building block pyridine 2,4-dicarboxylic acid by engineering recombinant *Pseudomonas putida* strains

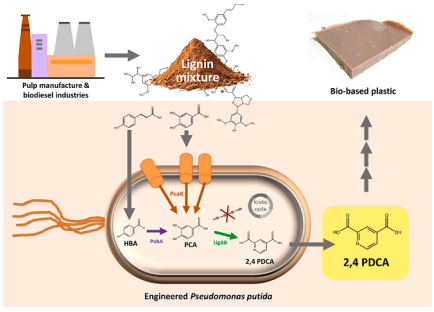
Helena Gómez-Álvarez [a,1], Pablo Iturbe [a,f,1], Virginia Rivero-Buceta [a], Paul Mines [b], Timothy D. H. Bugg [c], Juan Nogales [d,e], Eduardo Díaz [a,e,*]

[a] Margarita Salas Center for Biological Research, Spanish National Research Council, Ramiro de Maeztu 9, 28040 Madrid, Spain
[b] Biome Bioplastics Ltd, North Road, Marchwood, Southampton SO40 4BL, UK
[c] Department of Chemistry, University of Warwick, Coventry CV4 7AL, United Kingdom
[d] National Centre for Biotechnology, Spanish National Research Council, Darwin 3, 28049 Madrid, Spain
[e] Interdisciplinary Platform for Sustainable Plastics towards a Circular Economy-Spanish National Research Council (SusPlast-CSIC), Madrid, Spain
[f] Navarrabiomed, University of Navarra, Irunlarrea 3, 31008 Pamplona, Spain

HIGHLIGHTS

- *P. putida* was engineered to metabolise monoaromatics into the formation of 2,4 PDCA.
- Additional gene copies of PcaK transporter or PobA hydroxylase improves productivity.
- 2,4 PDCA production in resting cells sorts out growth-coupled bottlenecks.
- 2,4 PDCA production from soda lignin was achieved for the first time in *Pseudomonas*.

GRAPHICAL ABSTRACT



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ABSTRACT

2,4 pyridine dicarboxylic acid (2,4 PDCA) is an analogue of terephthalate, and hence a target chemical in the field of bio-based plastics. Here, *Pseudomonas putida* KT2440 strains were engineered to efficiently drive the metabolism of lignin-derived monoaromatics towards 2,4 PDCA in a resting cells-based bioprocess that alleviates growth-coupled limitations and allows biocatalysts recycling. Native β-ketoadipate pathway was blocked by replacing protocatechuate 3,4-dioxygenase by the exogenous LigAB extradiol dioxygenase. Overexpression of *pcaK* encoding a transporter increased 8-fold 2,4 PDCA productivity from protocatechuate, reaching the highest value reported so far (0.58 g $L^{-1}h^{-1}$). Overexpression of the 4-hydroxybenzoate monooxygenase (*pobA*) speed up drastically the production of 2,4 PDCA from 4-hydroxybenzoate (0.056 g $L^{-1}h^{-1}$) or *p*-coumarate (0.012 g $L^{-1}h^{-1}$) achieving values 15-fold higher than those reported with *Rhodococcus jostii* biocatalysts. 2,4 PDCA was also

* Corresponding author at: Microbial and Plant Biotechnology Department, Margarita Salas Center for Biological Research, Spanish National Research Council, Ramiro de Maeztu 9, 28040 Madrid, Spain.
E-mail address: ediaz@cib.csic.es (E. Díaz).
[1] These authors contributed equally to this work.

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bioproduced by using soda lignin as feedstock, paving the way for future polymeric lignin valorization approaches.

1. Introduction

Circular economy should aim for a more efficient fate of raw materials or feedstocks not only through recycling of the finally generated goods but also through the reincorporation of industrial, domestic wastes and residuals into the production-consumption cycle. Plant biomass, for instance, the substrate of pulp manufacture and biodiesel industries is only partially exploited. While the carbohydrate part (cellulose and hemicellulose) is used for paper, nylon or biofuel production, among other uses, the aromatic component (lignin) is extracted in amounts higher than 50 Mt per year worldwide (Bajwa et al., 2019) and is mostly considered a residue. Only part of it is used for macromolecular applications (production of glues, adsorbents or raw material for carbon nanofibers) (Chung and Washburn, 2013; Esposito and Antonietti, 2015; Liu et al., 2015; Norberg et al., 2013; Sen et al., 2015; Upton and Kasko, 2016) and most of it is combusted for heat and energy production within factory facilities (Liu et al., 2015). Lignin represents, however, the most abundant renewable source of aromatics. As demonstrated in several publications, lignin streams can be revalorized through green biotechnology using genetically modified bacteria for the production of compounds of undisputable commercial necessity and interest such as muconic acid (Chen et al., 2021; Salvachúa et al., 2018), adipic acid (Vardon et al., 2015), pyridine dicarboxylic acids (Johnson et al., 2019; Spence et al., 2021) or vanillin (Sainsbury et al., 2013), among others.

Production of bioplastics monomers synthesized from renewable feedstocks is of special relevance given the environmental concern that arises from petrochemical-based ones that depend on a non-sustainable feedstock. It is noteworthy to point out that plastics are produced in the order of hundreds of square tons per year worldwide, and only part of them (4Mt) are bio-based or biodegradable nowadays (Geyer et al., 2017). Pyridine dicarboxylic acids (PDCA), i.e., 2,4 PDCA and 2,5 PDCA, are chemical analogs of terephthalic acid, the main aromatic constituent of petrochemical-based plastics such as polyethylene terephthalate (PET) or polybutyrate adipate terephthalate (PBAT), and their biological synthesis represents an interesting alternative to the use of petro-based terephthalic acid for the production of drop-in bio-based plastics, e.g., PBAP (Pellis et al., 2019). These compounds have been previously produced in engineered *Rhodococcus jostii* RHA1 strains by re-routing the native protocatechuic acid (PCA) *ortho*-cleavage metabolism towards an implanted foreign PCA *meta*-cleavage, followed by ammonia cyclisation of the extradiol ring fission product (Mycroft et al., 2015; Spence et al., 2021). By using purified substrates like vanillic acid or 4-hydroxybenzoate (HBA), or by using the monoaromatics present in lignocellulose or soda lignin samples, maximum titers of 2,4 PDCA formation of about 1.5–3 mM (0.23–0.48 g/L) were reported in the culture medium of a recombinant *R. jostii* strain. Production of 2,5 PDCA in *Pseudomonas putida* KT2440 using HBA as substrate was reported with a maximum titer of about 3.6 mM (0.6 g/L) after 48 h bacterial growth (Johnson et al., 2019). Despite the use of *P. putida* cells enhanced for about 5-fold the PDCA productivity (g $L^{-1}h^{-1}$) with respect to those values obtained with *Rhodococcus* cells, the yield of the bioconversion process (mol PDCA/mol HBA) was significantly lower in *P. putida* (3.8%) than in *Rhodococcus* (about 50%). Moreover, no production of 2,4 PDCA has been described so far in a gram-negative microbial chassis despite of the fact that gram-negative bacteria, e.g. *Pseudomonas* strains, might be a suitable alternative to gram-positive biocatalysts since they are usually more solvent-tolerant and amenable to genetic manipulation (Ramos et al., 2002)

The non phatogenic soil bacterium *P. putida* KT2440 is one of the most promising platform organisms for lignin valorization (Johnson et al., 2016; Kumar et al., 2021; Notonier et al., 2021; Weimer et al., 2020; Yaguchi et al., 2021). In this work, a successful biotransformation process uncoupled to growth, i.e., a resting cell process, is presented in which, by using genetically engineered *P. putida* KT2440 strains, bacterial metabolism of lignin-derived compounds such as PCA, HBA or *p*-coumaric acid (pCA) is re-routed into 2,4 PDCA production (Fig. 1). Biotransformation yields and productivities were improved by accelerating substrates consumption and metabolism. Efficient 2,4 PDCA bioproduction was also shown from a real soda lignin feedstock.

2. Materials and methods

2.1. Chemicals, bacterial strains, plasmids and growth conditions

All chemicals were purchased from Merck unless otherwise stated. Green Value Protobind 1000 soda lignin (GVPL) was originally purchased from Green Value SA (Orbe, Switzerland) (Spence et al., 2021). Stock solution of 3% (w/v) GVPL was prepared as follows: lignin was initially dissolved in water by adding NaOH until pH 11.5 was reached. HCl was then used to lower pH to 8, and the solution was then autoclaved and kept at room temperature. 0.1 M stock solutions of aromatic substrates pCA and PCA, were prepared in water after neutralization (pH 7.5) of the corresponding acids with equimolar amounts of NaOH, and stored at −20 °C. In the case of HBA, 0.1 M stocks solutions were prepared by directly dissolving (without NaOH addition) sodium 4-hydroxybenzoate in water. Bacterial strains and plasmids used in this work are detailed in Table 1. *P. putida* KT2440 strains were cultivated in liquid lysogeny broth (LB) medium (Sambrook and Russell, 2001) or in M63 or M9 minimal media (Miller, 1972) with the specified carbon sources in each case. *E. coli* strains were cultivated in LB medium at 37 °C and 200 rpm agitation. When required, gentamicin (10 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (30 μg/ml) were added to the growth medium.

2.2. Molecular biology techniques.

Standard molecular biology techniques were performed as previously described (Sambrook and Russell, 2001). Plasmid DNA extractions were performed using a "High Pure plasmid isolation kit" (Roche Applied Science). DNA fragments were purified from agarose gels or PCR mixtures with "Gene Extraction Kit" or "PCR purification Kit" (Qiagen), respectively. Oligonucleotides employed (see supplementary material) were supplied by Sigma. 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 (Sambrook and Russell, 2001). Plasmids were transferred to *P. putida* KT2440 cells by triparental filter mating (Martínez-García et al., 2017) or electroporation (Gene Pulser, Bio-Rad). Triparental filter mating was performed using *E. coli* DH10B (pK18_ligAB) as donor strain, *E. coli* HB101 (pRK600) as helper strain and *P. putida* KT2440 as recipient strain.

2.3. Construction of the P. putida KT::ligAB recombinant strain

P. putida KT::ligAB strain (Table 1) was constructed by substituting the wild-type chromosomal *pcaG* gene of strain KT2440 with the *ligAB* genes from *Sphingobium* sp. SYK-6 through a double homologous recombination event using the pK18_ligAB suicide plasmid (Table 1). This plasmid bears a $lacI^q$ gene and a *Ptac* promoter that drives the inducible expression of the *ligAB* genes, whose sequence was codon-optimized for *P. putida* and a bicistronic Shine-Dalgarno sequence, BCD2 (Mutalik et al., 2013), was engineered to improve LigAB protein

production levels (see supplementary material). The BCD2-*ligAB* fragment was synthesized by GeneMill company (Liverpool). pK18_ligAB was delivered into *P. putida* KT2440 by triparental filter mating. Exconjugants resulting from the first site recombination event were selected on kanamycin-cetrimide (Sigma-Aldrich) plates containing 1 % glycerol as sole carbon source. Second site recombination event was selected by growth on M63-medium plates containing 0.2% citrate and 5% sucrose. Correct allelic exchange in sucrose-resistant and kanamycin-sensitive derivatives was validated by PCR using the two primers pairs ligAB_Fw_pK18/pcaH_Rv and lacIq_Rv/trmA_Fw. Both PCR products were sequenced to confirm the right construction of the recombinant *P. putida* KT::ligAB strain.

2.4. Cloning and expression of pcaK and pobA genes

The *pcaK* gene (PP_1376) was PCR amplified from *P. putida* KT2440 genomic DNA with primers pcaK_Fw_XbaI and pcaK_Rv_SacI (see supplementary material) and cloned into XbaI and SacI double-digested pIZ2 vector, generating plasmid pLasP8 (Table 1). A similar strategy was used for cloning the *pobA* gene (PP_3537) after amplification with pobA_Fw_SpeI_SD and pobA_Rv_HindIII primers (see supplementary material) and insertion into SpeI and HindIII double-digested pIZ2, generating plasmid pLasH42 (Table 1). In both cases, gene expression is driven by the *lacI*^{q}/*Ptac* regulatory couple. Plasmids carrying *pcaK* or *pobA* were transferred to *P. putida* KT::ligAB by electroporation, and gene expression was induced by adding 0.5 mM IPTG.

2.5. 2,4 PDCA production coupled to bacterial growth

P. putida KT::ligAB was grown in LB medium and 0.5 mM IPTG. After 3 h incubation at 200 rpm and 30 °C, cell pellets were used to inoculate M9 minimal medium containing 0.5 mM IPTG, 50 mM NH_4Cl, 1 mM PCA and 0.4 % (w/v) glucose as carbon source. During cultivation, 1 mL-supernatant samples were collected over 5 days, filtered through 0.45 μm pore size filters and stored at −20 °C for HPLC analysis.

2.6. 2,4 PDCA production in P. putida resting cells

1 mL of an overnight seed-culture of *P. putida* KT2440::ligAB strain containing control plasmid pIZ2, or plasmids pLasP8 (*pcaK*) or pLasH42 (*pobA*), grown in LB was used to inoculate 200 mL of fresh LB medium and incubated during 3 h at 30 °C. 0.5 mM of IPTG was then added to induce expression of *ligAB* genes, and also that of *pcaK* or *pobA* in strains harboring plasmids pLasP8 and pLasH42, respectively, followed by an additional 3 h incubation period to reach an OD_{600} of about 1.8. Cells were collected and resuspended in 10 mL of resting cells medium: 50 mM sodium phosphate buffer pH 7.5 supplemented with 0.1 M ammonium chloride and the aromatic substrate (optimized concentrations were 5 mM PCA, 1 mM HBA, 1 mM pCA, or 1.5 % (w/v) GVPL). These resting-cells mixtures (0.1 g dry cell weight) 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 HPLC analyses of metabolites accumulated

Table 1
Bacterial strains and plasmids used in this study.

Bacterial strains	Relevant genotype	Reference or source
E. coli DH10B	F, *mcrA* Δ*(mrr hsdRMS-mcrBC)* ϕ*80dlac*ΔM15 Δ*lacX74 deoR recA1 araD*139 Δ*(ara-leu)7697 galU galK*$^\lambda$ *rpsL endA1 nupG*	Invitrogen
E. coli HB101	Mating helper strain. F–, *thi*-1, *hsdS*20 (r_B–, m_B–), *sup*E44, *rec*A13, *ara*-14, *leu*B6, *pro*A2, *lac*Y1, *gal*K2, *rps*L20 (str^R), *xyl*-5, *mtl*-1	(Sambrook and Russell, 2001)
P. putida KT2440	Wild-type strain	(Bagdasarian et al., 1981)
P. putida KT::ligAB	KT2440 derivative with the chromosomal *pcaG* gene replaced by *ligAB* genes (Δ*pcaG::ligAB)*	This work
Plasmids	**Relevant genotype**	**Reference or source**
pK18mobsacB	Km^R. *ori*ColE1, Mob$^+$, *lacZα*; suicide vector with a *sacB* selection marker for gene replacement by double-site homologous recombination	(Schäfer et al., 1994)
pK18_ligAB	Km^R. pK18mobsacB vector bearing a *lacI*^{q}-*Ptac*- bicistronic Shine Dalgarno- *ligAB* genes fragment flanked by two DNA regions for homologous recombination leading to Δ*pcaG::ligAB* substitution in *P. putida* KT2440 chromosome.	This work
pRK600	Cm^R. Helper plasmid used for conjugation; *oriV*(ColE1), RK2(*mob* + *tra* +).	(Kessler et al., 1992)
pIZ2	Gm^R. pIZ1016 derivative with an extended polylinker.	(Acedos et al., 2021)
pLasP8	Gm^R. pIZ2 vector expressing *pcaK* from the *lacI*^{q}/*Ptac* regulatory couple.	This work
pLasH42	Gm^R. pIZ2 vector expressing *pobA* from the *lacI*^{q}/*Ptac* regulatory couple.	This work

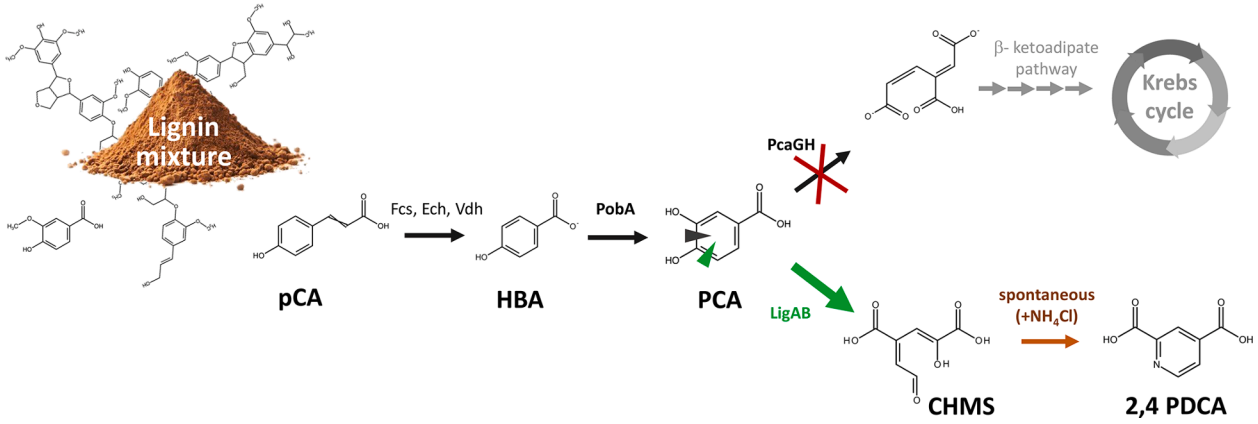

Fig. 1. 2,4 PDCA production from lignin-derived monoaromatics. Monoaromatics like *p*-coumaric acid (pCA) are present in some lignin mixtures. In *P. putida* KT2440, pCA is metabolized to 4-hydroxybenzoate (HBA) by action of Fcs, Ech, Vdh enzymes, and then to the central intermediate protocatechuate (PCA) by action of the PobA monooxygenase. In this work, the native (PcaGH-dependent) *ortho*-cleavage reaction of PCA is replaced by an exogenous (LigAB-dependent) *meta*-cleavage (green arrow), rendering 4-carboxy-2-hydroxymuconate-semialdehyde (CHMS). After spontaneous cyclisation with ammonium, the terephthalate analog 2,4-pyridine dicarboxylic acid (2,4 PDCA) is generated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in the supernatant. When using GVPL, supernatants were directly stored at −20 °C for later extraction with dichloromethane and HPLC analysis.

2.7. Metabolite analyses

To monitor 2,4 PDCA production from aromatic substrates, i.e., pCA, 4HBA or PCA, supernatant samples collected were acidified with 0.25 M HCl and directly analyzed by HPLC in an Agilent Technologies 1260 Infinity II machine using a C18 ZORBAX Eclipse Plus (95 Å, 4.6 × 250 mm, 5 μm, Agilent) reverse phase column. 2,4 PDCA, 4-carboxy-2-hydroxymuconate-semialdehyde (CHMS), and pCA were detected and quantified at 280 nm in a diode array type of UV/VIS detector (DAD); HBA and PCA were detected and quantified at 260 nm. Solvents used for HPLC analyses were: water/ 0.1% trifluoroacetic acid (solvent A) and methanol/ 0.1% trifluoroacetic acid (solvent B). The applied gradient for analysis was 15–50% of solvent B for 11 min followed by two column re-equilibration steps: 50–15% B for 1 min plus 15% B for 4 min. Flow rate was always kept at 1 mL/min. Chromatograms generated from samples were compared to that of authentic standards.

For HPLC analysis of samples derived from GVPL an extraction step with one volume of dichloromethane followed by vigorous vortexing and centrifugation at 13000 rpm for 5 min, was added. The upper aqueous phase was recovered, acidified with 0.25 M HCl and again vortexed and centrifuged. Pellets (containing precipitable polymeric lignin) were discarded and supernatants used for HPLC analysis, using the same column and equipment as described above. The HPLC elution conditions were: solvents A (water) and B (methanol) were acidified with 0.1% formic acid, flow rate was kept at 0.5 mL/ min and the applied B-solvent gradients were 10 min of 15–50%, followed by 10 min of 50–70% and a final 10 min re-equilibration step of 70–15%.

HPLC-MS (high-performance liquid chromatography coupled to mass spectroscopy) analyses of the supernatant samples were also performed. 2,4-PDCA, pCA, ferulic acid, and 4-carboxy-2-hydroxymuconate-semialdehyde (CHMS hemiacetal form) were detected by extracted ion analysis for fragments *m/z* 168, 165, 194, and 186, respectively, in positive ion mode, and were compared with the same profiles of authentic standards. The analysis was performed using a HPLC Surveyor and a Thermo Mod. Finnigan TM LXQ TM ion trap spectroscopy system and the column already mentioned.

3. Results and discussion

3.1. Re-routing the P. putida KT2440 β-ketoadipate pathway for 2,4 PDCA production in resting cells

In *P. putida* KT2440, catabolism of a variety of lignin-derived monomers present in lignocelluloses mixtures, e.g., pCA, coniferyl alcohol or ferulic acid (Linger et al., 2014; Salvachúa et al., 2015), is channeled to the central intermediate PCA, which is further metabolized through the β-ketoadipate pathway to finally yield acetyl-CoA and fuel the Krebs cycle (Jiménez et al., 2002). To re-route PCA towards 2,4 PDCA, a derivative *P. putida* KT2440 strain was generated by replacing the native *ortho*-cleavage 3,4 protocatechuate dioxygenase activity (*pcaGH* genes) by a heterologous *meta*-cleavage 4,5 protocatechuate dioxygenase coded by the *ligAB* genes from *Sphingobium* sp. SYK-6 (Fig. 1) (Kasai et al., 2005; Noda et al., 1990). As expected, this new strain (*P. putida* KT::ligAB, Table 1) lost the ability to use certain aromatic compounds that funnel to PCA, e.g., HBA or vanillate, as sole carbon sources since the bacterium cannot metabolize the PCA *meta*-cleavage product.

2,4 PDCA production coupled to growth was checked in *P. putida* KT::ligAB strain in minimal medium containing 0.4 % (w/v) glucose as carbon source and 1 mM PCA as substrate for the bioconversion. However, only 0.4 mM PCA was consumed and 0.05 mM 2,4 PDCA was produced at the end of the growth curve. This result indicated that the conversion yield (5%) was very limited and suggested that the PCA-*meta* cleavage product, a highly reactive semialdehyde compound (Fig. 1; see also below), could cause some toxicity problems in actively growing *P. putida* cells being challenging for fermentative production at high concentrations. Since it is well-known that resting (nongrowing) cells are suitable to produce growth-inhibitory or toxic chemicals, among other benefits (Sun et al., 2018; Dvořák et al., 2020; Zhou et al., 2020), it was envisioned that the bioconversion with *P. putida* KT::ligAB in a resting cells process could be particularly useful. To this end, *P. putida* KT::ligAB cells were collected after growth in LB medium and resuspended in resting cells buffer containing 5 mM PCA or 1 mM HBA. Supernatant samples were collected at different time points to assess the biotransformation time course. In contrast to growing cells experiments, complete consumption of substrates was accomplished (Fig. 2A and 2B). The 2,4 PDCA productivity from PCA and HBA was about 0.069 g $L^{-1}h^{-1}$ and 0.011 g $L^{-1}h^{-1}$, respectively, which was higher than that previously reported from HBA (0.003 g $L^{-1}h^{-1}$) in growing cells of *R. jostii* (Mycroft et al., 2015; Spence et al., 2021). Therefore, these results revealed for the first time that a resting cell process is a suitable strategy to efficiently produce 2,4 PDCA with the *P. putida* KT::ligAB recombinant biocatalyst, leading to both a significant reduction of the bioconversion times and a significant increase of the bioconversion efficiency with respect to growing-cells processes.

3.2. Detection of a PCA ring-cleavage co-product that limits 2,4 PDCA yield

Although it was shown above that resting cells of *P. putida* KT::ligAB are able to convert the aromatic substrate added, less than 50 % yield for 2,4 PDCA production was achieved (Fig. 2A and 2B), suggesting the accumulation of an additional product(s). It is known that LigAB dioxygenase cleaves PCA generating a yellow intermediate, 4-carboxy-2-hydroxymuconate-semialdehyde (CHMS), that spontaneously cycles into 2,4 PDCA in the presence of ammonium (Fig. 3A) (Mycroft et al., 2015). However, CHMS can as well cycle spontaneously into its hemiacetal form (closed form), being the open and closed forms in a pH-dependent equilibrium (Barry and Taylor, 2013). At neutral or acidic pH the hemiacetal has been demonstrated to be the predominant form, and the open form (CHMS) is found in the case of basic pH (Fig. 3A). In spite of the high concentration of ammonium (0.1 M) supplied in the biotransformation reactions (pH 7.5), the presence of a peak (elution time 6.5 min) in the HPLC chromatograms of similar signal intensity to that of 2,4 PDCA (elution time 8 min) (Fig. 3A) was observed. This peak at 6.5 min matches the predicted *m/z* (186) of CHMS (Fig. 3A) and becomes of an intense yellow color under alkaline conditions (Fig. 3B), strongly suggesting that about half of the PCA *meta*-cleavage product accumulates as the hemiacetal form and, hence, limits the 2,4 PDCA yield to values below 50%. Efforts were done to try to prevent accumulation of the hemiacetal form, e.g., use of higher amounts of ammonium in the biotransformation process, use of higher pH (up to 9) to promote the presence of the open form and thus a more efficient cyclisation towards 2,4 PDCA, but so far none of them increased the 2,4 PDCA yield.

The generation of CHMS in parallel to 2,4 PDCA production might pose some kind of distress for cells during growth. Previous attempts to produce 2,4 PDCA using a modified *P. putida* strain (where *pcaHG* was substituted by *ligAB*) in a 10-L bioreactor with glucose as carbon source and HBA as substrate did not reveal a significant accumulation of PDCA while the aromatic substrate or the PCA intermediate were continuously accumulating (Johnson et al., 2019). However, production of PDC (2-pyrone-4,6-dicarboxylic acid) coupled to the enzymatic dehydrogenation of CHMS catalyzed by LigC using a *P. putida* strain with an additional modification (substitution of *pcaHG* with *ligAB* and *ligC* genes) growing in glucose and HBA reached a titer of 58 g/L (Johnson et al., 2019). These results point out that lack of CHMS production and the accumulation of substrates in the *P. putida* strain expressing *ligAB* are not related to carbon catabolite phenomena affecting transport or

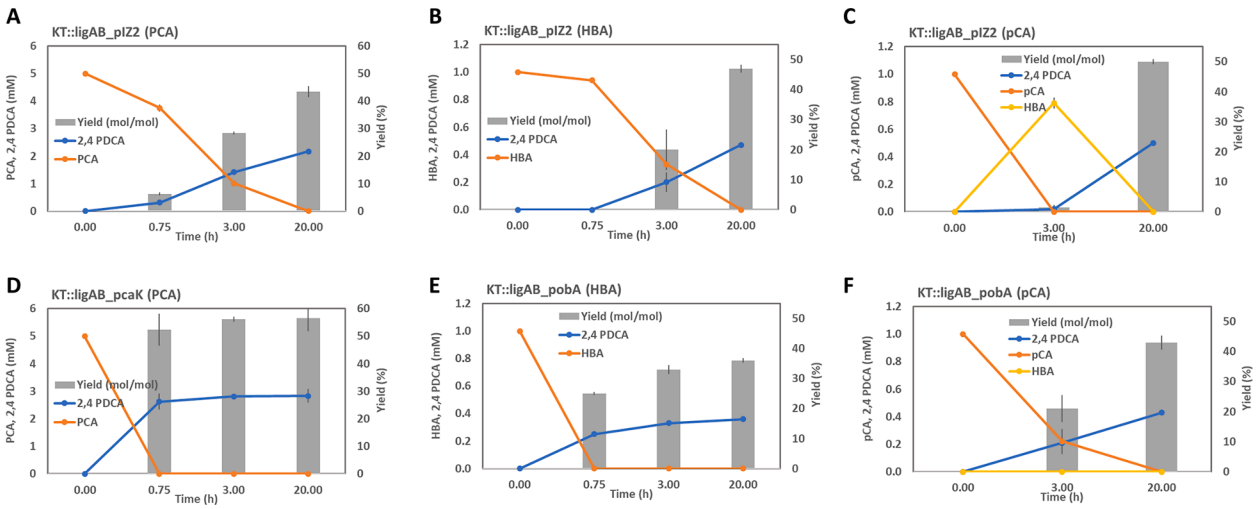

Fig. 2. 2,4 PDCA production over time from PCA, HBA or pCA in the *P. putida* KT::ligAB derivative strains bearing either an empty pIZ2 plasmid (KT::ligAB) or the pLasP8 (KT::ligAB/pcaK) or pLasH42 (KT::ligAB/pobA) plasmids. The results shown represent the mean and standard deviation (error bars) of three independent resting cells experiments performed as detailed in section 2.6. The conversion yield is shown with grey bars.

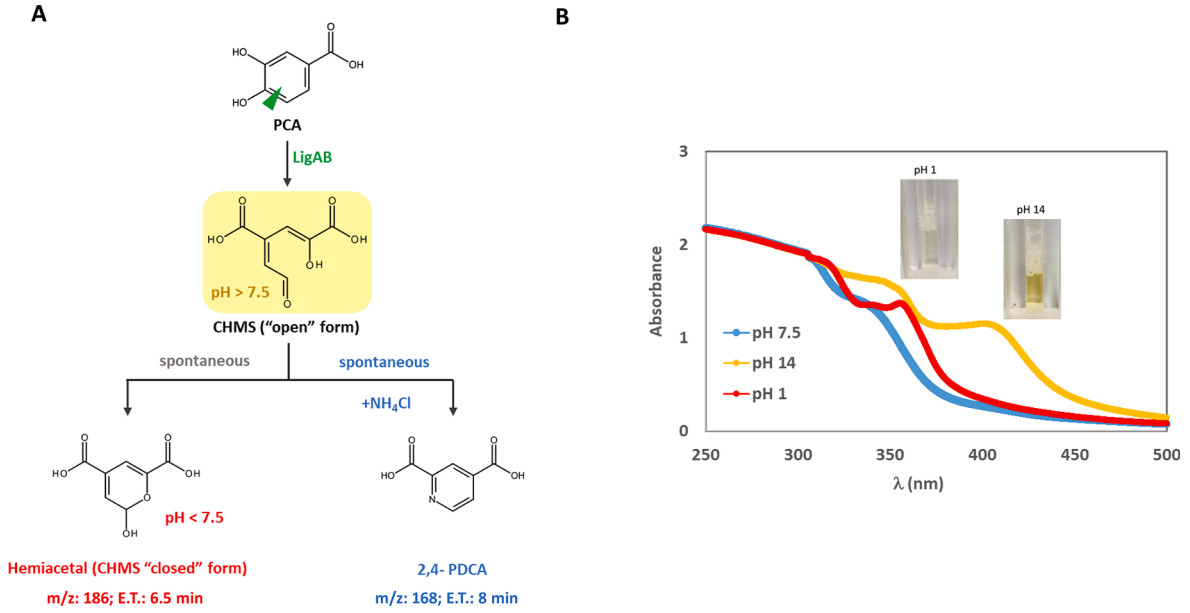

Fig. 3. (A) Scheme of PCA *4,5-meta* cleavage by LigAB and the formation of CHMS (open form, yellow compound), hemiacetal (CHMS closed form) and 2,4 PDCA. The HPLC elution time (E.T.) and MS mass/charge ratio (*m/z*) of CHMS and 2,4 PDCA are indicated. (B) UV–Vis spectra of the same biotransformation products at neutral (blue), acidic (red) or alkaline (yellow) pH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

metabolism of the aromatic substrates nor to the lack of optimum culture conditions, but to toxicity exerted by CHMS that is generated but not further metabolized. This hypothesis would also explain why CHMS, and thus 2,4 PDCA, could be generated in the present work in resting cells conditions but were very poorly accumulated in growing cells conditions.

3.3. Improvement of 2,4 PDCA production efficiency from PCA by overexpression of a PcaK transporter

PCA can be biologically synthesized (Li and Ye, 2021) and it is a relevant substrate for the industrial production of PDCAs (Law et al., 2016). It has been shown above that *P. putida* KT::ligAB is able to convert PCA into 2,4 PDCA in a resting cells-based process. However, the uptake of highly hydroxylated aromatics, such as PCA, inside the bacterial cells has been shown as a major bottleneck limiting the efficiency of some biotechnological processes. In this sense, the expression of genes encoding aromatic transporters was shown to improve the bioproduction of target metabolites (Mori et al., 2018; Wu et al., 2018). Therefore, it was checked whether the regulated overexpression of a PCA transporter, i.e., the *pcaK* gene (PP_1376) (Harwood et al., 1994; Wada et al., 2021), in the *P. putida* KT::ligAB strain could enhance further the 2,4 PDCA production efficiency. As shown in Fig. 2A, *P. putida* KT::ligAB cells consumed 25% PCA after 45 min of the resting cell process, leading to a 6% 2,4 PDCA yield and 0.07 g $L^{-1}h^{-1}$ productivity. However, cells that overexpress the PcaK transporter (*P. putida* KT::ligAB containing plasmid pLasP8; Table 1) after induction (IPTG addition) resulted in a complete consumption of the substrate PCA, generating 2,4 PDCA at a 52% yield (Fig. 2D) and with an almost ten-fold increase of volumetric productivity (0.58 g $L^{-1}h^{-1}$), the highest

reported so far for the bioproduction of 2,4 PDCA. Therefore, these results demonstrate that the regulated overexpression of the PcaK transporter is a successful strategy to speed up 2,4 PDCA production when *P. putida* KT::ligAB resting cells use PCA as aromatic substrate, and they suggest that increasing expression levels of aromatic transporters may be also a key factor when developing resting cells-based bioprocesses feed by hydrophilic substrates.

It is worth noting that *P. putida* KT::ligAB (pLasP8) resting cells could be recycled and used in a further cycle of PCA conversion to 2,4 PDCA maintaining the same yield and volumetric productivity than in the first cycle and hence allowing a 2,4 PDCA titer up to 0.9 g/L (5,4 mM) (Fig. 4A). This cell recycling strategy may not only facilitate higher 2,4 PDCA production but also reduce process costs, being another advantage of using resting cells in this bioconversion process.

3.4. Improvement of 2,4 PDCA production efficiency from lignin-derived monoaromatics by overexpression of 4-hydroxybenzoate hydroxylase (PobA)

Hydroxycinnamic acids, e.g. pCA and ferulic acid, are lignin components in grasses and they can be released from the aromatic polymer by several physico-chemical treatments (Linger et al., 2014). Since *P. putida* KT2440 can degrade pCA through a peripheral pathway (*fcs/ech/vdh* genes) generating HBA which then becomes hydroxylated to PCA as central intermediate (Fig. 1), it was checked whether pCA could be converted into 2,4 PDCA in *P. putida* KT::ligAB resting cells. As shown in Fig. 2C, whereas a minor amount of pCA (less than 1%) was converted into 2,4 PDCA after 3 h incubation time, a significant amount (79 %) was excreted as HBA. Long incubation times resulted in a complete consumption of pCA with no accumulation of HBA and 50% yield of 2,4 PDCA (Fig. 2C). This transient accumulation of HBA during the metabolism of pCA has been also observed during production of muconic acid by engineered *P. putida* KT2440 growing cells (Rodriguez et al., 2017), and it was partially reduced by removing the *crc* gene encoding the global regulator of carbon catabolite repression (Johnson et al., 2017).

The transient accumulation of HBA during the bioconversion of pCA in *P. putida* KT::ligAB resting cells suggested that hydroxylation of HBA to form PCA was a major bottleneck during the initial bioconversion of pCA to 2,4 PDCA. To try to overcome this problem and speed up the bioconversion process, the *pobA* gene (PP_3537) encoding the monooxygenase that converts HBA into PCA (Jiménez et al., 2002) was overexpressed under control of the *lacI*^{q}/*Ptac* regulatory couple in the *P. putida* KT::ligAB strain containing plasmid pLasH42 (Table 1). In contrast to the *P. putida* KT::ligAB strain, the strain overexpressing *pobA* consumed almost 80% pCA without accumulation of HBA at time 3 h, and the 2,4 PDCA yield was about 21% with a productivity of 0.012 g L^{-1}h^{-1} (Fig. 2F). A similar improvement of 2,4 PDCA production was observed at short incubation times when using HBA as substrate, reaching a productivity value of 0.056 g L^{-1}h^{-1} (Fig. 2B and 2E), a value that is more than 15 times higher than that reported with growing cells of recombinant *R. jostii* (0.003 g L^{-1}h^{-1}) (Spence et al., 2021).

In summary, all these results show that overexpression of *pobA* in *P. putida* KT::ligAB cells significantly enhances the production of 2,4 PDCA when using pCA and HBA as substrates, and it should be regarded as a promising strategy when designing recombinant *Pseudomonas* biocatalysts to valorize lignin samples containing these aromatics.

In addition, it was shown that the *P. putida* KT::ligAB (pLasH42) biocatalyst could be recycled for at least two biotransformation cycles without losing performance, reaching a titer of 0,13 g/L (0,7 mM) 2,4 PDCA (Fig. 4B).

3.5. Production of 2,4 PDCA from a lignin sample

The results presented above indicate that *P. putida* KT::ligAB recombinant biocatalysts are able to synthesize 2,4 PDCA from purified lignin-derived monoaromatic compounds added to the resting cells assays. To check whether the biocatalysts could be also able to generate 2,4 PDCA when treated with a real lignin sample, 1.5% (w/v) Green Value Protobind lignin (GVPL) was used, a commercially available soda lignin prepared from wheat straw/sarkanda. This lignin has been structurally characterized as a S/G/H lignin containing preferentially β-O-4 units, and some hydroxycinnamic acids (ferulic and *p*-coumaric acids) attached to the lignin polymer via ester and ether linkages (Constant et al., 2016; Williamson et al., 2020). GVPL was incubated with *P. putida* KT::ligAB (pLasH42) resting cells and 2,4 PDCA formation was analyzed by HPLC and confirmed by MS (see supplementary material) after extraction with dichloromethane (Fig. 5). Interestingly, the amount of 2,4 PDCA produced, i.e., around 0.04 g/L (0.24 mM), was in good agreement with the observed decrease of the peaks of pCA and ferulic acid, both of which become released from GVPL in the autoclaved culture medium (Williamson et al., 2020) (see supplementary material) and are metabolized to PCA by the *P. putida* cells. The addition of an external amount of pCA (1 mM) to GVPL lead to a corresponding increase of the 2,4 PDCA production (Fig. 5 and supplementary material), which confirms that the bioconversion process occurs efficiently in the presence of polymeric lignin.

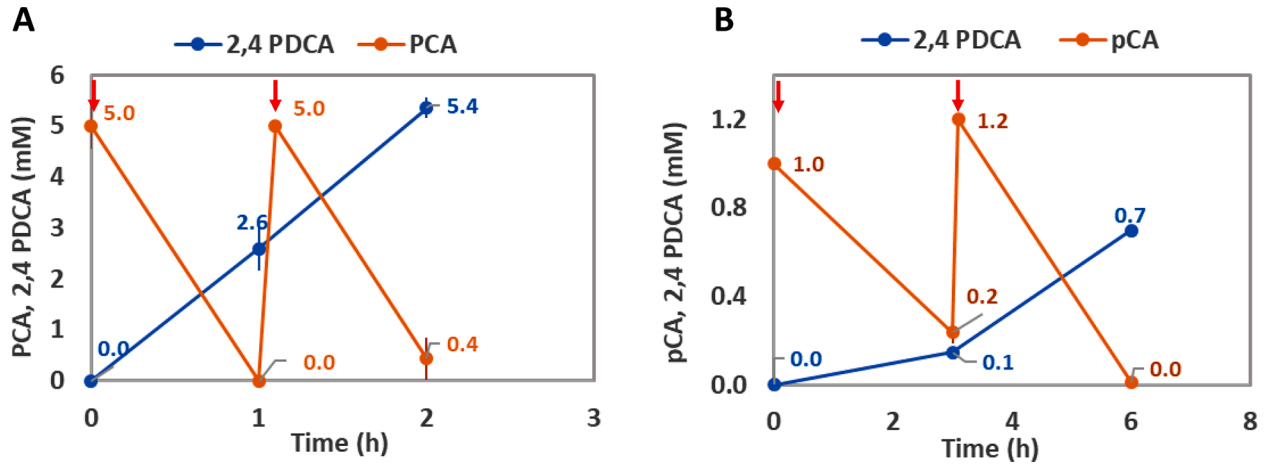

Fig. 4. 2,4 PDCA production by substrate pulse additions to resting cells. The results shown represent the mean and standard deviation (error bars) of three independent resting cells experiments performed as detailed in section 2.6. Bioconversions were performed with strain *P. putida KT*::ligAB containing plasmid pLasP8 (*pcaK)* in the presence of 5 mM PCA (A) or with strain *P. putida* KT::ligAB containing plasmid pLasH42 (*pobA)* in the presence of 1 mM pCA (B). Substrate additions are indicated with red arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

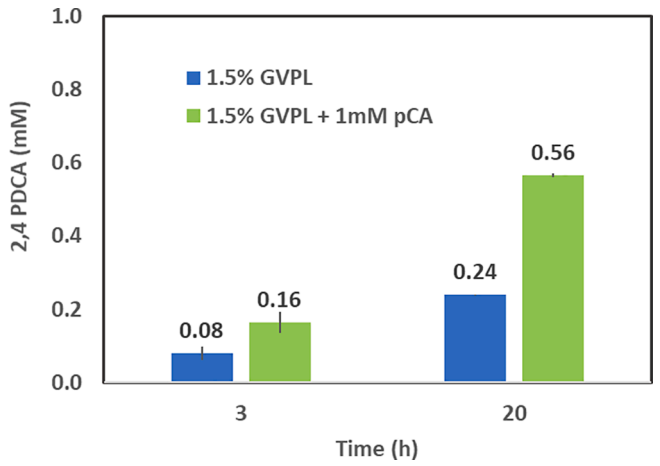

Fig. 5. Time course of 2,4 PDCA production from polymeric lignin. 1.5 % polymeric lignin, either GVPL alone (blue bars) or supplemented with 1 mM pCA (green bars), was treated with *P. putida* KT::ligAB (pLasH42) biocatalysts. The results shown represent the mean and standard deviation (error bars) of three independent resting cells experiments as detailed in section 2.6. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

For the first time the production of 2,4 PDCA in *P. putida* recombinant strains has been shown. An efficient resting cells-based bioprocess that alleviates growth-coupled limitations and allows biocatalysts recycling has been developed. Overexpression of the PCA transporter (*pcaK*) or the HBA monooxygenase (*pobA*) proved to increase 2,4 PDCA productivity from PCA (0.58 g $L^{-1}h^{-1}$) or HBA (0.056 g $L^{-1}h^{-1}$), reaching values more than 15 times higher than those reported with *R. jostii. pobA* overexpression also boosted 2,4 PDCA productivity from lignin-derived compounds, e.g., pCA, and allowed the use of polymeric lignin for 2,4 PDCA bioproduction.

CRediT authorship contribution statement

Helena Gómez-Álvarez: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Pablo Iturbe:** Conceptualization, Methodology. **Virginia Rivero-Buceta:** Methodology. **Paul Mines:** Conceptualization. **Timothy D.H. Bugg:** Conceptualization, Resources. **Juan Nogales:** Conceptualization, Writing – review & editing. **Eduardo Díaz:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2021.126638.

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