microbial biotechnology

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Synthesis of aromatic amino acids from 2G lignocellulosic substrates

Patricia Godoy,[1] Ana García-Franco,[1] María-Isabel Recio,[1,2] Juan-Luis Ramos[1,*] and Estrella Duque[1]

[1]*Consejo Superior de Investigaciones Científicas, Estación Experimental del Zaidín, c/Profesor Albareda 1, Granada, 18008, Spain.*

[2]*BioEnterprise Master Program, School of Pharmacy, University of Granada, Granada, Spain.*

Summary

Pseudomonas putida **is a highly solvent-resistant microorganism and useful chassis for the production of value-added compounds from lignocellulosic residues, in particular aromatic compounds that are made from phenylalanine. The use of these agricultural residues requires a two-step treatment to release the components of the polysaccharides of cellulose and hemicellulose as monomeric sugars, the most abundant monomers being glucose and xylose. Pan-genomic studies have shown that *Pseudomonas putida* metabolizes glucose through three convergent pathways to yield 6-phosphogluconate and subsequently metabolizes it through the Entner–Doudoroff pathway, but the strains do not degrade xylose. The valorization of both sugars is critical from the point of view of economic viability of the process. For this reason, a *P. putida* strain was endowed with the ability to metabolize xylose via the xylose isomerase pathway, by incorporating heterologous catabolic genes that convert this C5 sugar into intermediates of the pentose phosphate cycle. In addition, the open reading frame T1E_2822, encoding glucose dehydrogenase, was knocked-out to avoid the production of the dead-end product xylonate. We generated a set of DOT-T1E-derived strains that metabolized glucose and xylose simultaneously in culture medium and that reached high cell density with generation times of around 100 min with glucose and around 300 min with xylose. The strains grew in 2G hydrolysates from diluted acid and steam explosion pretreated corn stover and sugarcane straw. During growth, the strains metabolized > 98% of glucose, > 96% xylose and > 85% acetic acid. In 2G hydrolysates *P. putida* 5PL, a DOT-T1E derivative strain that carries up to five independent mutations to avoid phenylalanine metabolism, accumulated this amino acid in the medium. We constructed *P. putida* 5PLΔ*gcd* (*xylABE*) that produced up to 250 mg l^{-1} of phenylalanine when grown in 2G pretreated corn stover or sugarcane straw. These results support as a proof of concept the potential of *P. putida* as a chassis for 2G processes.**

Received 10 March, 2021; revised 4 May, 2021; accepted 13 May, 2021.

For correspondence. *E-mail juanluis.ramos@eez.csic.es; Tel. 34-958181600; Fax 34-958181608.

Microbial Biotechnology (2021) **14**(5), 1931–1943
doi:10.1111/1751-7915.13844
Funding information
MINECO (Grant / Award Number: 'RTI2018-094370-B-I00').

Introduction

Society is in favour of replacing polluting fossil fuels (the main source of energy), and oil-derived chemicals with alternative renewable sources to combat global climate change (Ragauskas *et al*., 2006; Valdivia *et al*., 2016). A variety of new green energy sources are currently being used (bio-, eolic, thermosolar and photovoltaic energy); however, the sole and main alternative for oil-derived chemicals today is a new 'green chemistry' based on plants as the only sustainable and renewable source of organic carbon on earth. This so-called new chemistry is expected to move the organic chemical industry towards net zero emissions (Ragauskas *et al*., 2006, 2014; Linger et al., 2014; Isikgor and Becer, 2015; Beckham *et al*., 2016; Ramos *et al*., 2016; Duque and Ramos, 2019).

At present, the major source of plant-derived materials to produce a number of chemicals is starch, whose hydrolysis yields glucose, that in turn is converted into different chemicals through fermentation processes. The use of starch to obtain biofuels and biochemical commodities is, however, controversial because of the overlap with the food chain. This controversy provoked a shift at the end of the last century when scientists began working on replacing starch by non-edible plant biomass such as lignocellulose, an attractive source of raw material due to its abundance and because it does not compete directly with foodstuffs. However, the use of lignocellulosic materials to produce bioethanol and biochemicals is challenging because biomass requires intensive pretreatment (mainly physico-chemical) to deconstruct it and for the release of cellulose,

hemicellulose and lignin (Alcántara *et al*., 2016). Sugars can be obtained from cellulose and hemicellulose in a process called saccharification, which involves enzymatic hydrolysis by a set of enzymes generically known as cellulases that work synergistically to produce monomeric sugars (Öhgren *et al*., 2007; Álvarez *et al*., 2016). Glucose (about 57–70%) is the major product that results from the hydrolysis, followed by xylose (about 9–23%), and other minority sugars such as arabinose, rhamnose and galactose (van Maris *et al*., 2006; Rocha-Martín *et al*., 2018; see Table S1 for data from Rocha-Martín *et al*., 2018). However, many industrially relevant microorganisms only ferment glucose and leave the other sugars untransformed. Techno-economic studies by Valdivia *et al*. (2016, 2020) revealed that the processing of the C5 xylose is a must in order to profitably produce bioethanol or other chemicals from biomass.

A number of xylose catabolic pathways have been engineered for heterologous expression in industrially relevant microorganisms. Recombinant xylose-utilizing strains of yeasts (García-Sanchez et al., 2010) and various bacteria like *Zymomonas mobilis* (Zhang *et al*., 1995), *Corynebacterium glutamicum* (Kawaguchi, *et al*., 2006), *Bacillus subtilis* (Chen *et al*., 2013; Zhang *et al*., 2016), *Kluyveromyces marxianus* (Suzuki *et al*., 2019) and *Pseudomonas putida* (Meijnen *et al*., 2008, 2009; Le Meur *et al*., 2012; Dvorák and de Lorenzo, 2018; Bator *et al*., 2020) have been constructed and can be considered as a first step towards efficient biomass utilization. An aim of synthetic biology is to establish a universal platform for the synthesis of 'all chemicals'. We are, however, far from that objective today and several microbial platforms based on recombinant microbes are being used to produce different chemicals (Calero and Nikel, 2019; Liu and Nielsen, 2019). Bioprocess yields depend on the metabolic route and on the stoichiometry of the pathways, as well as on the intrinsic properties of the chemicals and the microbial tolerance to the high concentrations of substrates and products that are demanded during the industrial production of commodities (Bator *et al*., 2020). We have focused our attention on the production of aromatic chemicals from sugars. To this end, we have chosen *P. putida* DOT-T1E as a production platform, because of its high resistance to a wide range of aromatic compounds, including aromatic hydrocarbons such as toluene, xylenes and styrene (Rojas *et al*., 2003; Ramos *et al*., 2015). This property confers significant advantage over other *P. putida* strains that are only moderately tolerant to solvents, for instance KT2440 (Segura *et al*., 2012).

DOT-T1E is a non-pathogenic strain of *Pseudomonas putida*. The pangenome of the species revealed a set of core genes common to all strains and which define the basic physiological properties of these microorganisms. The species has also a set of accessory genes that are present in only two or more but not all the strains of the species, which confer specific properties (e.g. biodegradation of lineal and/or aromatic hydrocarbons), and the ability to colonize different niches (Udaondo *et al*., 2016). The pangenome showed that the species *P. putida* is characterized by a limited ability to consume sugars, that is glucose, gluconate and fructose, which are mainly metabolized through the Entner–Doudoroff pathway (Nelson *et al*., 2002; del Castillo *et al*., 2007; Daddaoua *et al*., 2009; Daniels *et al*., 2010; Tiso *et al*., 2014; Calero and Nikel, 2019). KEGG analysis revealed that *P. putida* DOT-T1E, similarly to other strains of the species, contains a set of genes that would allow xylose catabolism if different peripheral modules are added to transform xylose into central metabolic intermediates. In fact, Bator *et al*. (2020) described that up to three peripheral xylose pathways can be implemented in *P. putida* KT2440 to allow growth on xylose. These pathways are known as the isomerase pathway and the oxidative Dahms and Weimberg pathways (Meijnen *et al*., 2008, 2009; Bator *et al*., 2020). Bator *et al*. (2020) analysed the biotransformation of xylose into 14 different chemicals by recombinant *P. putida* strains and showed that the maximal product yields of 12 of the 14 metabolites of xylose were produced by the isomerase pathway. In this metabolic pathway, xylulose-5-phosphate is part of the pentose phosphate cycle and is eventually transformed into erythrose-4-phosphate, a starting metabolite for the synthesis of aromatic amino acids via the shikimate pathway (Fig. 1).

The isomerase pathway is characterized by the presence of a xylose isomerase (XylA) that transforms xylose into xylulose and a xylulokinase (XylB) that subsequently phosphorylates the latter to xylulose-5-phosphate, which enters into the Pentose Phosphate (PP) cycle (Wilhelm and Hollenberg, 1985; Amore *et al*., 1989; Mishra and Singh, 1993; Hahn-Hägerdal and Pamment, 2004). The *xylA* and *xylB* genes from the isomerase pathway of *E. coli* have already been engineered into KT2440 and S12 strains to allow the use of xylose (Le Meur *et al*., 2012; Dvorák and de Lorenzo, 2018). In both strains, the efficient use of xylose as a C source requires the inactivation of glucose dehydrogenase (Gcd) to avoid the misrouting of xylose to dead-end xylonate. While S12 was able to grow on xylose upon incorporation of *xylAB* (Meijnen *et al*., 2008), Dvorák and de Lorenzo (2018) and Elmore *et al*. (2020) described that KT2440 requires, in addition to *xylAB*, the *xylE* gene encoding a proton-coupled symporter to facilitate the entry of xylose into the cell.

This study shows that the isomerase pathway together with the *xylE* gene can be expressed in both *P. putida*

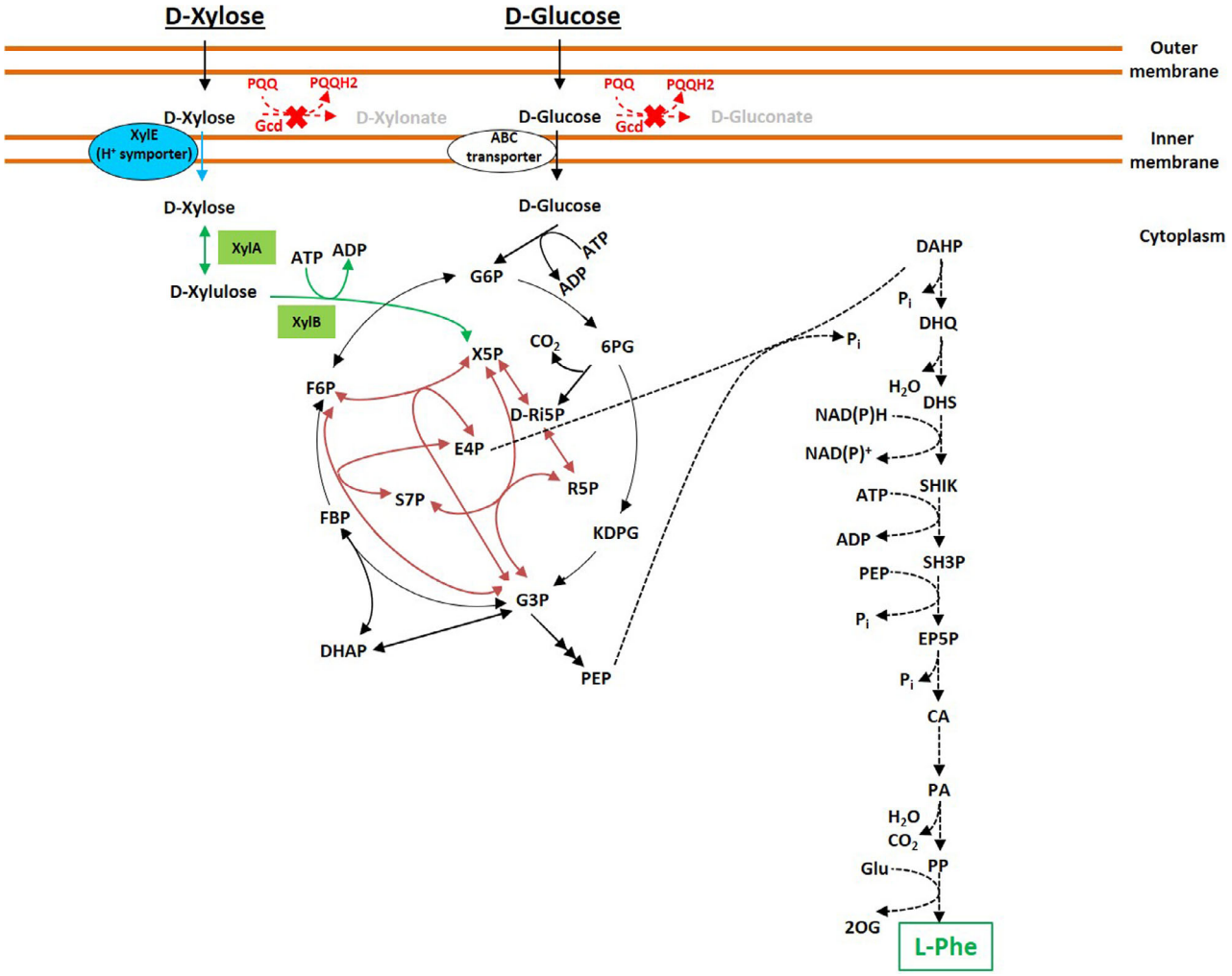

Fig. 1. Proposed synthesis of L-phenylalanine (L-Phe) from glucose and xylose in an engineered *P. putida* Δ*gcd* (*xylABE*) strain. Exogenous xylose transporter (blue) and xylose isomerase pathway enzymes (green) are highlighted. Green arrows indicate the exogenously supplemented xylose isomerase pathway. Brown arrows indicate native pentose phosphate pathway reactions. Dotted black arrows indicate native shikimate pathway reactions. Dotted red arrows indicate the glucose dehydrogenase (*gcd*) deleted reaction. Abbreviations: PQQ, pyrroloquinoline; G6P, D-glucose-6-phosphate; 6PG, 6-phosphogluconate; KDPG, 2-dehydro-3-deoxyphosphogluconate; G3P, D-glyceraldehyde 3-phosphate; FBP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; X5P, xylulose-5-phosphate; D-Ri5P, L-ribulose-5-phosphate; R5P, ribose-5-phosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrose 4-phosphate; PEP, phosphoenolpyruvate; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; SHIK, shikimate; SH3P, shikimate 3-phosphate; EP5P, 5-enolpyruvoylshikimate 3-phosphate; CA, chorismate; PA, prephenate; PP, phenylpyruvate; Glu, glutamate; 2OG, 2-oxoglutarate; L-Phe, L-Phenylalanine.

DOT-T1E wild-type strain and its glucose dehydrogenase (*gcd*) mutant derivatives. The recombinant strains not only grew on xylose in minimal medium, but also on corn stover and sugarcane straw 2G hydrolysates, consuming >98% glucose and >96% xylose. Furthermore, *P. putida* 5PL, a mutant derivative of DOT-T1E that overproduces *L*-phenylalanine (Molina-Santiago *et al*., 2016), was also engineered to inactivate the *gcd* gene and transformed with pSEVA633_*xylABE*. The resulting 5PLΔ*gcd* (*xylABE*) strain produced *L*-phenylalanine from 2G hydrolysates confirming the potential of *Pseudomonas putida* as a chassis to produce value-added goods from agricultural residues.

Results

Construction of Pseudomonas putida *DOT-T1E derivatives that use xylose and produce* L*-phenylalanine*

Pseudomonas putida DOT-T1E (Table 1) does not use xylose as a carbon source (Table 2); however, when DOT-T1E is incubated with xylose, it consumes the sugar and stoichiometrically converts it into the dead-

Table 1. Strains and plasmids used in this study.

Strains/plasmids	Genotype or relevant characteristics	Reference
Escherichia coli		
DH5α	Cloning host: F-λ-*endA1 glnX44*(AS) *thiE1 recA1 relA1 spoT1 gyrA96*(NalR) *rfbC1 deoR nupG* Φ80(*lacZ*ΔM15) Δ(*argF-lac*)*U169 hsdR17*($r_K^-m_K^+$)	Hanahan (1985)
CC118λpir	Cloning host: *araD139* Δ*(ara-leu)7697* Δ*lacX74 galE galK phoA20 thi– 1 rpsE rpoB*(Rif^R) *argE* (Am) *recA1*, λpir lysogen	Herrero *et al.* (1990)
Pseudomonas putida		
DOT-T1E	Prototroph, Cm^R, Rif^R	Ramos *et al.* (1995)
DOT-T1EΔ*gcd*	DOT-T1E mutant generated by insertional inactivation of DOT-T1E_2882, Km^R	This work
DOT-T1E (*xylABE)*	Wild type harbouring plasmid pSEVA633	This work
DOT-T1EΔ*gcd* (*xylABE)*	DOT-T1E Δgcd mutant harbouring plasmid pSEVA633_*xylABE*	This work
5PL	DOT-T1E mutant that produces *L*-phenylalanine	Molina-Santiago *et al.* (2016)
5PLΔ*gcd*	5PL mutant generated by insertional inactivation of DOT-T1E_2882, Km^R	This work
5PL (*xylABE)*	5PL harbouring plasmid pSEVA633_*xylABE*	This work
5PLΔ*gcd (xylABE)*	5PL Δ*gcd* harbouring plasmid pSEVA633_*xylABE*	This work
Plasmids		
pMBL-T	PCR product cloning vector, Ap^R	Canvax Biotech
pBBRMCS5	Broad-host-range, cloning vector, Gm^R	Kovach *et al.* (1995)
pBBRMCS5_*xylAB*	pBBRMCS5 bearing *xylAB* genes	This work
pSEVA2213_*xylABE*	pSEVA2213 expression vector bearing *xylABE* genes, Km^R	Dvorák and de Lorenzo (2018)
pSEVA633	Expression vector: *oriV*(pBBR1), *lacZ*α-pUC18, Gm^R	Silva-Rocha *et al.* (2013)
pSEVA633_*xylABE*	pSEVA233 with *xylABE* genes expressed from EM7 promoter	This work
pMBL-T:: T1E_2822ΩKm	pMBL-T- bearing the DOT-T1E-2822 and the ΩKm cassette. Ap^R, Km^R	This work
pHP45ΩKm	Source of a Km resistance cassette, Km^R	Fellay *et al.* (1987)

Table 2. Growth parameters of different *P. putida* strains on sugars.

	Glucose			Xylose			Glucose + Xylose		
Strain	tg	Y	Q	tg	Y	Q	tg	Y	Q
DOTDOT-T1E	102	0.35	0.25	n.g	n.g	n.d.	102	0.29	(0.17 + 0.06)
DOT-T1EΔ*gcd*	102	0.36	0.25	n.g	n.g	n.d.	96	0.34	(0.17 + 0.00)
DOT-T1E (*xylABE)*	101	0.35	0.24	299	0.32	0.22	100	0.35	(0.17 + 0.06)
DOT-T1EΔ*gcd* (*xylABE)*	105	0.37	0.24	288	0.40	0.22	101	0.39	(0.17 + 0.06)
5PL	105	0.26	0.22	n.g	n.g	n.d.	94	0.21	(0.18 + 0.06)
5PLΔ*gcd*	115	0.25	0.22	n.g	n.g	n.d.	112	0.22	(0.18 + 0.00)
5PL *(xylABE)*	99	0.38	0.22	279	0.39	0.22	102	0.36	(0.18 + 0.06)
5PLΔ*gcd* (*xylABE)*	100	0.39	0.22	274	0.40	0.22	102	0.35	(0.18 + 0.06)

tg, generation time expressed in min; Y, yield expressed as g cells g^{-1} sugar consumed within 24 h; Q, sugar consumption rate expressed in g $l^{-1}{\cdot}h^{-1}$. For glucose + xylose assay the first value correspond to glucose consumption while the second one corresponds to xylose consumption rate; n.g, no growth; n.d, not determined.

Growth conditions and analytical determination are described in detail under Experimental procedures. The data in the Table are the average of at least two independent assays done each in triplicate. Standard deviations are below 10% of the given values.

end product xylonate (data not shown). In other *P. putida* strains, xylonate synthesis proceeds in the periplasm in a reaction catalysed by glucose dehydrogenase, encoded by the *gcd* gene (T1E_2822). A DOT-T1E-derivative strain devoid of this gene was constructed as described in the Experimental Procedures section (Table 1). This mutant did not transform xylose into xylonate (data not shown), suggesting that Gcd is the only apparent enzyme involved in xylose biotransformation in DOT-T1E. As described in the Introduction, conversion of xylose into xylulose-5-phosphate – an intermediate of the PP cycle – allows metabolism of xylose as a C-source in other *Pseudomonas* strains (Dvorák and de Lorenzo, 2018; Elmore *et al.*, 2020). To channel xylose towards PP cycle intermediates, we amplified the *xylAB* genes from *E. coli* (see Experimental Procedures), cloned them into pBBRMCS5 which carries the gene encoding gentamycin resistance (Table 1), and transformed the recombinant derivative into the wild-type DOT-T1E and its Δ*gcd* isogenic mutant (Table 1;

Fig. S1). The transformants were spread on M9 minimal medium plates supplied with gentamycin and glucose or xylose as a C source; no transformants able to grow on xylose were found, while the rate of gentamycin resistant (Gm^R) transformants on glucose was 10^4 clones per μg DNA. None of the glucose selected clones were able to grow on xylose in spite of long-term incubation. This result contrasts with those observed in *P. putida* S12 bearing the *xylAB* genes, which acquired xylose degradation capacity upon enrichment in xylose (Meijnen *et al*., 2008). However, they are in line with the results observed in KT2440, which revealed that the mere incorporation of the catabolic genes was not sufficient to allow growth of this strain on xylose (Dvorák and de Lorenzo, 2018). These authors showed that, in addition, a xylose symporter was needed to facilitate uptake of xylose and its metabolism (Dvorák and de Lorenzo, 2018; Elmore *et al*., 2020; Espeso *et al*., 2021). To address this, we subcloned the synthetic *xylABE* operon designed by Dvorák and de Lorenzo (2018) into the Gm^R pSEVA633 plasmid. (This construction was made to use a Gm resistant cassette compatible with the Km-resistant marker present in the Δ*gcd* strains). DOT-T1E and DOT-T1EΔ*gcd* bearing *xylABE* constructs were selected on Gm containing M9 minimal plates with glucose as the sole C-source. These clones grew when replicated onto Gm containing M9 minimal plates with xylose, although at a slower rate than on glucose.

We then used liquid culture medium to compare the growth rates, growth yields and sugar consumption rates of DOT-T1E, DOT-T1EΔ*gcd*, DOT-T1E (*xylABE*) and DOT-T1EΔ*gcd* (*xylABE*) on glucose, xylose and mixtures of glucose and xylose (Table 2). We found that growth of DOT-T1E and DOT-T1EΔ*gcd* on minimal medium with glucose was similar, reaching a turbidity at 660 nm of around 4 and ~ 10^{10} CFU ml^{-1}. Doubling times were about 100 min (i.e. see Fig. S2), and yields of both strains on glucose were in the order of 0.35 to 0.36 g dry weight g^{-1} glucose consumed (Table 2). This indicated that the lack of the *gcd* gene did not significantly affect the utilization of glucose in these strains. In fact, the rate of carbon consumption of both strains was 0.25 g l^{-1} h^{-1}. The two strains bearing the *xylABE* genes grew on glucose at a similar rate (101–105 min doubling time) to the strains without pSEVA633_*xylABE*, suggesting that the *xylABE* gene load does not influence growth rate.

On xylose, DOT-T1E (*xylABE*) and DOT-T1EΔ*gcd (xylABE)* strains grew to reach 10^9 to 10^{10} CFU ml^{-1}, with doubling times of about 300 min (Table 2), and yields in the range of 0.32 to 0.40 g dry weight g^{-1} xylose consumed. The rate of xylose consumption was around 0.22 g l^{-1} h^{-1}. Similarly to other *P. putida* strains in which the *xyl* isomerase pathway had been engineered, our recombinant strains also metabolized xylose via the PP cycle. Elmore *et al*. (2020) showed that increased expression levels of two genes (*tal* and *tkt*) were needed for efficient channelling of xylose through the PP cycle in KT2440. However, this might not be the case with DOT-T1E, because high growth rates, high xylose consumption rates and high yields were obtained without exogenous increases in *tal* and *tkt* expression. The growth parameters and C consumption rates we achieved with DOT-T1E derivatives were slightly higher than those described for KT2440 engineered to use xylose (Dvorák and de Lorenzo, 2018).

As the major sugars in 2G hydrolysates are glucose and xylose, we compared the growth characteristics on glucose plus xylose (at a ratio 3:1) of DOT-T1E and DOT-T1EΔ*gcd* with and without *xylABE* genes (Table 2). As expected, with mixtures of glucose and xylose, all clones grew with similar doubling times (Table 2). For the two clones that used xylose as a C-source, we found that cultures reached stationary phase after 24 h without apparent diauxic growth. In fact, time course glucose and xylose consumption with the DOT-T1E (*xylABE*) and DOT-T1E*Δgcd* (*xylABE*) strains revealed simultaneous utilization of both sugars from the beginning, with glucose assimilation rates of around 0.17 mmol l^{-1} h^{-1} for both strains while xylose was assimilated at a rate of 0.06 g l^{-1} h^{-1}. Therefore, C5 sugar utilization was not under carbon catabolite control in the recombinant *P. putida* strains. The yields of the strains using glucose and xylose were in the range of 0.29 to 0.39 g dry weight per g sugar consumed (Table 2).

Previous comparative analysis based on yields and flux balances in *Pseudomonas* strains indicated that the best pathway for synthesis of added value chemicals is dependent on the stoichiometry of the reactions (Bator *et al*., 2020) and these authors concluded that the isomerase pathway is the most favourable. We have previously described that the DOT-T1E 5PL mutant strain was able to produce *L*-phenylalanine from glucose (Molina-Santiago *et al*., 2016). The strain was engineered to block phenylalanine catabolism and bore a $pheA^{br}$ mutation. The $pheA^{br}$ allele encodes a variant of the enzyme that catalyses the Claysen rearrangement of chorismate to prephenate and its ulterior conversion into phenylpyruvate, the first step in phenylalanine biosynthesis, the mutant $PheA^{br}$ is not susceptible to feed-back inhibition due to accumulation of phenylalanine.

Then, strain 5PL was considered a potential useful chassis for aromatic amino acid production from 2G sugars. To explore this, we introduced the *xylABE* operon into 5PL and its isogenic Δ*gcd* mutant. We tested growth of 5PL (*xylABE*) and 5PL*Δgcd* (*xylABE)* on glucose, xylose and a mixture of both sugars (Table 2). The 5PL*Δgcd* (*xylABE)* and 5PL (*xylABE)* strains grew with glucose until

cell densities of 10^9–10^{10} CFU ml^{-1}, with doubling times in the range of 100–115 min, and growth yields of 0.25–0.39 g dry weight per g glucose, respectively (Table 2). Carbon consumption rates of 5PL (*xylABE)* and 5PL*Δgcd* (*xylABE)* with xylose were similar to those achieved by equivalent constructions in DOT-T1E, even though they only grew linearly, but reaching cell densities in the order of 10^9–10^{10} CFU ml^{-1}. Growth rates, growth yields and C-consumption rates in the presence of glucose and xylose were similar to those measured with only glucose. We analysed *L*-phenylalanine production from glucose and xylose by 5PL Δ*gcd* (*xylABE)* and found that production was higher with glucose (225 ppm) than with xylose (10 ppm), but that the amount of *L*-phenylalanine produced with mixtures of both sugars was similar to that measured with glucose (Table 3). The limited production of *L*-phenylalanine from xylose arose not only from the limited uptake of the carbon source, but also from limited synthesis of shikimate, because exogenous addition of 1 mM shikimate to a xylose metabolizing strain enhanced *L*-phenylalanine production to 75 ppm (Table 3). In agreement with this series of results, the level of *L*-phenylalanine produced with glucose plus xylose was lower than with only glucose and approached those reached with only glucose (Table 3). This supports the idea that although xylose is directly channelled into the PP cycle, the level of PP cycle intermediates was higher with glucose than with xylose.

We envisaged that we would see improvements in the *L*-phenylalanine production pathway through manipulation of *aro* genes, as has been described for *L*-phenylalanine production in *E. coli* (Liu *et al*., 2018) and aromatic derivatives in *P. putida* (Loeschcke and Thies, 2020; Schwanemann *et al*., 2020). In any case, our set of results support that *P. putida* bearing the *xylABE* genes is a potentially useful platform for metabolism and biotransformation of 2G sugars into added value chemicals.

Table 3. Phenylalanine accumulation from 2G sugars by *P. putida* 5PLΔ*gcd* (*xylABE*).

C-source	Phenylalanine (mg l^{-1})
Glucose	225
Glucose + shikimate (1mM)	230
Xylose	10
Xylose + shikimate (1mM)	75
Glucose plus xylose	175
Glucose plus xylose + shikimate (1mM)	211

Growth conditions and analytical determination are described in detail under Experimental procedures. The data are the average of at least two independent assays. Phenylalanine concentration in the culture medium was determined at the start of the assay (concentration was negligible in all cases) and after 24 h incubation (given values in the Table). Standard deviations are below 15% of the given values.

Growth of Pseudomonas putida *DOT-T1E strain on 2G substrates derived from corn stover and sugarcane straw*

Because the ultimate aim of this work is to explore the potential of *Pseudomonas putida* as a platform to produce added value chemicals from 2G substrates, we decided to test the behaviour of the set of strains constructed in this study in hydrolysates of 2G lignocellulosic material upon saccharification. The lignocellulosic material we choose was pretreated acid-diluted and steam exploded corn stover (PCS) and sugarcane straw (PSCS). PCS and PSCS were prepared at the York (Iowa, USA) laboratory of Abengoa Bioenergy. Figure 2 top shows the structure of a corn stover and sugarcane stalk with a set of fibrils in the centre that includes the xylem and phloem tubes, characteristic of these plants. Upon steam explosion in acidic medium, the cell material was found to be disorganized and long fibres appeared (Fig. 2 bottom). The composition for PCS (Table S1) is about 35% glucan as cellulose, 7.8% xylan-hemicellulose, 3% soluble glucose/cellobiose and 16% soluble xylose (Rocha-Martín *et al*., 2018). For PSCS, the composition was glucan-cellulose 36%, 4.5% xylan-hemicellulose, 3% soluble glucose/cellobiose and 8.2% soluble xylose (Rocha-Martín *et al*., 2018).

To release monomeric sugars from the polymers, a suspension of 10% (w/v) of the pretreated lignocellulosic materials was digested for 24 h with different amounts of the commercial cellulase cocktail Viscozyme (Sigma-Merck) at pH 5.5–6.0 in 1xM9 solution incubated at 50 °C with moderate agitation (50–70 rpm in an orbital shaker). Upon digestion with Viscozyme, we found that the highest glucose yield was about 6.7 g glucose l^{-1} and 3 g xylose l^{-1} in the case of PCS, and 5.8 g kg^{-1} glucose and 1.8 g xylose l^{-1} in the case of PSCS (see Tables S2 and S3). The hydrolysates were neutralized to pH 7 with phosphate buffer and used undiluted or diluted to 5%, 3% and 1% substrate as a C-source. These suspensions were inoculated with DOT-T1E, DOT-T1E (*xylABE*), DOT-T1E*Δgcd* and DOT-T1E*Δgcd* (*xylABE*) to reach an initial cell density of 10^6–10^7 CFU ml^{-1}. We found that at a load of 10% of 2G hydrolysates from PCS or PSCS, none of the strains grew in this medium; however, with lower substrate loads, cell density in the 2G PCS and PSCS hydrolysates reached between $3x10^8$ and $7x10^9$ CFU ml^{-1}. The results shown in Table 4 reveal that with a substrate charge of 3% (w/v) PSC or PSCS, all strains were able to consume more than 99% of glucose and more than 70% of the xylose in 24 h (>96% in 48 h), except for

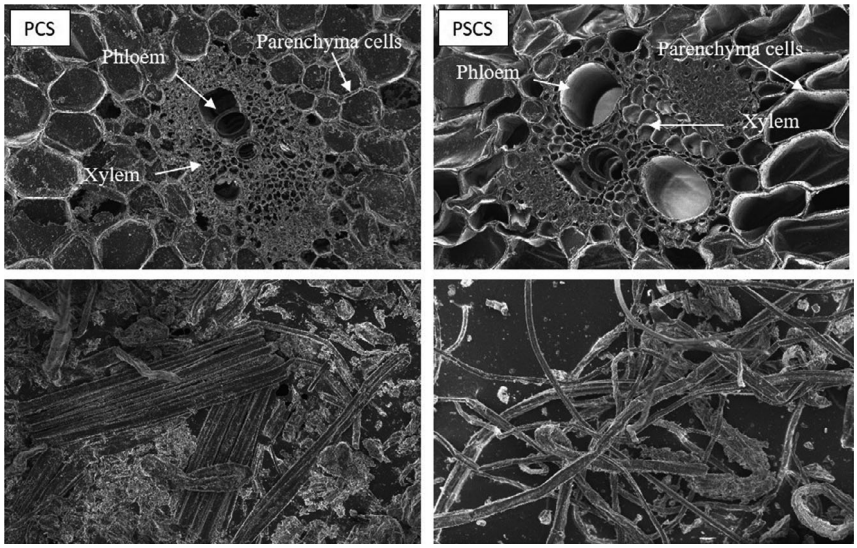

Fig. 2. Scanning electron microscopy (SEM) of pretreated or untreated agricultural wastes. Top left, corn stover (100x); top right (500x), and sugarcane straw (PSCS) or bottom left (100x) and bottom right (500x) images of these materials after steam explosion in the presence of diluted acid.

Table 4. Utilization of 2G substrates by *Pseudomonas putida* derivatives and growth.

	G_0	G_{24}	X_0	X_{24}	$AcOH_0$	$AcOH_{24}$	CFU $ml^{-1}{}_0$	CFU $ml^{-1}{}_{24}$
3% PSCS								
T1E	1550	10	1780	60	260	18	3.53E+07	2.49E+09
T1EΔ*gcd*	1175	9	1424	1450	230	19	1.18E+08	3.48E+09
T1E (*xylABE)*	1230	0	897	1	223	9	3.17E+07	2.14E+09
T1EΔ*gcd* (*xylABE)*	1060	0	1300	13	210	3	6.17E+07	5.33E+09
3% PCS								
T1E	1630	22	2770	505	232	34	1.55E+07	2.98E+09
T1EΔ*gcd*	1445	0	3050	3090	205	12	1.74E+08	4.40E+09
T1E (*xylABE)*	1405	7	2810	4	202	13	5.67E+07	2.72E+09
T1EΔ*gcd* (*xylABE)*	1490	0	2665	8	211	7	5.50E+07	3.17E+09

Assay conditions are described under experimental procedures. The data in the table are the average of at least two assays.
Concentration of substrates is in mg l^{-1}. Standard deviations were below 15% of the given values.
X_0: Xylose in solution at the start of the assay; X_{24}: Xylose in solution after 24 h incubation.
G_0: Glucose in solution at the start of the assay; G_{24}: Glucose in solution after 24 h incubation.
$AcOH_0$: Acetate in solution at the start of the assay. $AcOH_{24}$: acetate in solution after 24 h incubation.
CFU_0: Colony forming units at the start of the assay; CFU_{24}: Colony forming units after 24 h incubation.

DOT-T1EΔ*gcd*, as expected, that did not consume xylose.

Elmore *et al*. (2020) reported that a KT2440 derivative carrying the *xylABE* genes was able to use xylose but did not assimilate arabinose, this contrasted with the results in S12 that uses xylose and arabinose upon acquisition of *xylAB* genes and adaptation to growth on xylose. We have not analysed arabinose consumption by T1E derivatives in detail, but in our 2G hydrolysates assays, we determined the levels of arabinose before and after growth of six recombinant strains and found that arabinose levels remain unchanged.

In addition, we determined if any of the strains inoculated in 2G hydrolysates would consume acetic acid present at concentration of 200–260 mg l^{-1}. We found that >85% of the initial acetic acid concentration was removed. This set of results suggests that the 2G hydrolysates from pretreated lignocellulosic material can be used for growth of *Pseudomonas* on 2G materials.

Similar assays to those described above with the DOT-T1E (*xylABE*) and its isogenic Δ*gcd* mutant were carried out with the 5PL (*xylABE)* and 5PLΔ*gcd* (*xylABE)* strains. We found that the strains grew to reach cell densities of 2 to 3 × 10^8 CFU ml^{-1} and accumulated up to

Table 5. Utilization of 2G substrates by *Pseudomonas putida* 5PL derivatives and biosynthesis of *L*-phenylalanine.

	G_0	G_{24}	X_0	X_{24}	A_0	A_{24}	Phe_0	Phe_{24}
PSCS								
5PL (*xylABE)*	1490	20	1950	54	230	15	0	200
5PLΔ*gcd (xylABE)*	1490	10	1950	75	230	4	0	250
PSC								
5PL (*xylABE)*	1780	10	3440	220	180	0	0	160
5PLΔ*gcd (xylABE)*	1780	30	3440	1560	180	22	0	220

Assay conditions are described under experimental procedures. The data in the table are the average of at least two assays.
Concentration of chemicals is in mg l^{-1}.
Standard deviations were below 15% of the given values.
X_0: Xylose in solution at the start of the assay; X_{24}: Xylose in solution after 24 h incubation.
G_0: Glucose in solution at the start of the assay; G_{24}: Glucose in solution after 24 h incubation.
$AcOH_0$: Acetate in solution at the start of the assay. $AcOH_{24}$: acetate in solution after 24 h incubation.
Phe_0: phenylalanine in solution at the start of the assay; Phe_{24}: phenylalanine in solution after 24 h incubation.

200–250 ppm *L*-phenylalanine in the hydrolysed supernatants (Table 5).

Discussion

2G cell factories and Pseudomonas

The increase in food prices due to the use of cereal grain in the production of so-called first-generation (1G) biofuels (including ethanol) led to the search of new sources of sugars for bioethanol production. The new source of raw material for the so-called 2G technology is biomass, whose abundance allows for sufficient global feedstock, that is in the United States alone, biomass could reach more than 450 Mdryton/year and this amount, in terms of bioethanol equivalence, is 67 Ggal ethanol/year (www.energy.gov/sites). Bioethanol prices are highly volatile and, as such, industry searches for new opportunities in the production of added value products from 2G hydrolysates.

Frank (2010) estimated that the economic potential of biochemicals produced from lignocellulosic residues could reach a value of ~$1 trillion USD, and that more than 60% of the world's most-used chemicals could be synthesized from lignocellulose. In spite of these promising prospects, over the last decade the number of ongoing large-/medium-scale biosynthetic processes that use biomass has remained rather limited (Aristodou and Penttilä, 2000; Chandel *et al*., 2018). Examples of chemicals produced from 2G hydrolysates at different scales are as follows: ethanol, lactic acid, succinic acid, butanol, acetone, sorbitol and itaconic acid (Valdivia *et al*., 2016; Chandel *et al*., 2018). We now add to this list, the production of phenylalanine, an aromatic amino acid that can be transformed into other chemicals such as *trans*-cinnamic acid and styrene (McKenna and Nielsen, 2011; Molina-Santiago *et al*., 2016). A number of Pseudomonads expressing heterologous genes produced surfactants, organic acids, terpenoids, phenazines and bioplastics from glucose (Rojas *et al*., 2003; Wittgens *et al*., 2011; Loeschcke and Thies, 2015; Tiso *et al*., 2017; Wittgens *et al*., 2018; Wynands *et al*., 2018). We envisage that the production of this set of chemicals from 2G sugars will be feasible once one of the xylose catabolic pathways described by Bator *et al*. (2020) is incorporated into these strains.

A true challenge in using 2G technology at the industrial level is not only the conversion of highly recalcitrant and low solubility lignocellulose into monomeric sugars but also their efficient use for chemical production. This requires a series of physico-chemical treatments to deconstruct plant structures and make cellulose and hemicellulose available for subsequent enzymatic hydrolysis to yield soluble sugars. Figure 2 shows that dilute-acid steam explosion pretreatment of herbaceous residues resulted in appropriate levels of biomass disorganization of corn stover and sugarcane straw. This specific pretreatment released about 30% to 50% of xylose in the polymers but less than 3% of the glucose (see Table S1 and Rocha-Martín *et al*., 2018). Subsequent action of cellulase cocktails can release up 80% of total sugars as monomers (Álvarez *et al*., 2016). In addition to sugars, the 2G hydrolysates contain acetic acid, furfural and lignin monomers. It is known that some of the chemicals generated from biomass during the pretreatment process are inhibitors of growth. Industrial production of biochemicals from 2G hydrolysates requires as high as possible load of 2G hydrolysates to warrant high production of commodities. The typical 2G bioethanol production processes operate at a solid ratio of 20% (Alcántara *et al*., 2016); but to generate economic returns, the yields should be close to the theoretical, which in the case of 2G bioethanol production requires that the strains of *Saccharomyces* used in the process should consume > 96% glucose and > 90% xylose (Valdivia *et al*., 2016).

Co-utilization of different sugars from lignocellulose by industrial microbes is complex and often subject to catabolite repression which most often prioritizes the use

of glucose over other sugars. We have found that this is not the case in our constructs as glucose and xylose are simultaneously consumed. In fact, *P. putida* derivatives bearing *xylABE* genes consumed >96% of the sugars released in the saccharification step. The rates of substrate utilization (0.22–0.3 g l^{-1} h^{-1}) were superior to those reported with engineered *Saccharomyces* to produce ethanol with 2G substrates (Heer and Sauer, 2008). This, in turn, resulted in the highest concentrations of *L*-phenylalanine being generated in quite a short time, which in industrial terms will lead to a reduction in the number of fermenters per plant, with a consequent saving in the initial investment, a relevant factor to make the 2G technology economically viable (Valdivia *et al*., 2016).

A current limitation in the use of *Pseudomonas* as a 2G platform is that *P. putida* growth in 2G hydrolysates was inhibited when the initial substrate loads were > 5% (w/v). Acetate – which is among the potential inhibitors of growth – seems not to be the responsible for *P. putida* growth inhibition, because the strain naturally uses acetic acid as a C source and consumed > 85% of the initial acetate in the 2G hydrolysates within 24 h. Other inhibitors present in 2G hydrosylates are furfural, and hydroxymethylfurfural, which are inhibitors of yeast growth (Heer and Sauer, 2008). Mutant strains of *Saccharomyces* tolerant to furfural and able to thrive in lignocellulose hydrolysates have been isolated. However, our preliminary results indicate that T1E tolerates concentrations of up to 20 mM furfural which is higher than those present in 2G hydrolysates; at present, we cannot discard the additive effects of different chemicals as being responsible for substrate toxicity. We are setting up a Laboratory Adaptation Evolution programme to select *P. putida* strains able to thrive in up to 20% (w/v) 2G hydrolysate load. These mutants are expected to be profitable not only for synthesis of *L*-phenylalanine, as determined in this study, but in general to exploit *P. putida* industrially to achieve high product concentrations from high initial substrate loads.

In summary, we show that *P. putida* DOT-T1E*Δgcd (xylABE)* is able to grow using the C-sources available in 2G hydrolysates. As *P. putida* can be easily manipulated to express heterologous pathways, it has high potential for further development as an industrial platform.

Experimental procedures

Bacterial strains, plasmids and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in LB medium, while *Pseudomonas putida* strains were grown in LB medium or in M9 minimal medium routinely with glucose 5 g l^{-1} (Abril *et al*., 1989). Liquid cultures were incubated in a Kühner incubator at 30 °C with agitation (200 rpm).

DNA techniques and plasmid and strain constructions

General techniques. DNA was manipulated using standard laboratory protocols (Sambrook and Russell, 2001). Genomic DNA was isolated using the Wizard Genomic DNA purification Kit (Promega USA), while plasmid DNA was isolated with the QIAprep Spin Miniprep kit (Qiagen, USA). DNA concentration was measured with a Nano Drop One^{C} (Thermo Scientific, USA). PCR DNA amplification was performed with appropriate primers (see Table S4), dNTPs and Q5 high fidelity DNA polymerase (New England BioLabs, USA) or Taq DNA polymerase (Roche, Germany), as recommended by the manufacturers.

Electroporation. Electroporation of *E. coli* DH5α and different *Pseudomonas putida* strains with ligation mixtures or complete plasmids was performed as described elsewhere (Aparicio *et al*., 2015), using a MicroPulser electroporator and Gene Pulser Cuvettes with 0.2 cm gap (Bio-Rad, USA). Transformants were selected on LB agar plates with Km (25 μg ml^{-1}) or Gm (10 μg ml^{-1}) incubated at 30 °C for 24 to 36 h.

Construction of xylose metabolic modules. The *xylAB* genes of the *E. coli xyl* operon encoding xylose isomerase (EC 5.3.1.5) XylA, and xylulokinase (EC 2.7.1.17) XylB, were amplified from genomic DNA of *E. coli* DH5α using xylAB-KpnI and xylAB-HindIII primers (Table S4). The PCR product was cloned into pMBL-T vector and recombinant plasmid was digested with KpnI and HindIII and ligated into pBBRMCS5 (Gm^{R}), giving rise to pBBRMCS5_*xylAB*.

A synthetic operon made of xylose isomerase (*xylA*), xylulokinase (*xylB*) and the xylose-proton symporter (*xylE*) from *E. coli* was recovered from plasmid pSEVA2213_*xylABE* (Dvorák and de Lorenzo, 2018), after digestion with PacI/HindIII. The fragment containing *xylABE* genes and the constitutive EM7 promoter was ligated into pSEVA633 to yield pSEVA633_*xylABE*.

Construction of mutants deficient in Gcd activity. Inactivation of T1E_2822 encoding glucose dehydrogenase (Gcd) was achieved as follows: a 1009 bp DNA fragment spanning the central part of this ORF was amplified by PCR from *P. putida* DOT-T1E genomic DNA using primers T1E_2822Fw and T1E_2822Rv (Table S4). The resulting amplified DNA was cloned into pMBL to yield pMBL::T1E_2822. This plasmid was then digested with BamHI and then ligated to the BamHI kanamycin Ω-interposon fragment from

plasmid pHP45Ω-Km. The resulting chimeric DNA was named pMBL::T1E_2822ΩKm. This plasmid was electroporated into *P. putida* DOT-T1E and *P. putida* 5PL, respectively, and putative Km^R recombinant mutants were selected on kanamycin LB plates. A number of Km^R strains were retained, and Southern blotting was used to verify the insertional mutation in the *gcd* gene (see Fig. S2). A *gcd* mutant strain from DOT-T1E and from 5PL parental strains, referred to as *P. putida* DOT-T1EΔ*gcd* and 5PLΔ*gcd*, respectively, were retained for further study (Fig. S2).

Growth parameters

For growth experiments with different carbohydrates, overnight cultures of *P. putida* were grown in M9 minimal medium with glucose as sole carbon source, harvested by centrifugation (13 000 *g*, 5 min) and washed once with M9 minimal medium (Abril *et al.*, 1989). Then, cells were suspended to an OD_{660} of ~0.1 in 30 ml of the test medium. When required, kanamycin (25 μg ml^{-1}) or gentamicin (10 μg ml^{-1}) were added. Doubling times were determined during exponential growth as a slope of the data points obtained by plotting CFU ml^{-1} or turbidity at 660 nm against time.

For cell dry weight (CDW) determination, samples of cultures grown in M9 minimal medium with 5 g l^{-1} carbon source, namely glucose, xylose or a mixture of glucose:xylose (3:1, v:v), were transferred into 2 ml preweighed Eppendorf tubes and pelleted at 13 000 *g* for 10 min. The pellets were washed once with 1xM9 buffer and left to dry at 70 °C for 48 h. Substrate consumption rates and specific carbon consumption rates (qs) were determined during the initial 24 h of culture as described (Dvorák and de Lorenzo, 2018).

Other analytical techniques

Cell growth was routinely monitored at 660 nm using a Perking Elmer Lambda 20 UV VIS spectrophotometer (USA). A turbidity of 1.0 is equivalent to 414 mg l^{-1} of cell dry weight (CDW).

For determination of metabolites, cultures (1 ml) were centrifuged (13 000 *g*, 4 °C, 10 min), and 0.5 ml of the supernatants was stored at −20 °C until analysed. For analysis of sugars and acetic acid, D-GLUCOSE-HK, K-XYLOSE, K-ARGA and K-ACETRM Assays Kits (Megazyme, Ireland) were used according to the manufacturer's instructions. Measurements were performed using a TECAN Sunrise 200 microplate absorbance reader (Tecan GmbH, Austria).

L-Phenylalanine levels were determined in culture supernatants using an Agilent/HP 1050 HPLC System (Agilent/HP, USA), equipped with a Nova-Pak C18 column (4 μm, 3.9 mm × 150 mm, Waters) and coupled to a DAD detector. MilliQ H_2O acidulated with 0.1% (v/v) H_3PO_4 (A) and acetonitrile:H_2O (90:10, v/v), also supplemented with 0.1% H_3PO_4 (B), were used as eluents. Samples (20 μl) were injected for analysis at a constant flow rate of 0.85 ml min^{-1} for isocratic separation using a mixture of 75% (v/v) A and 25% (v/v) B. When an elution gradient was required, we used the same eluents with the following ramp of solvents and times: Method started with 2 min 100% A; then, mobile phase changed to 30% B within 8 min, followed by a 2 min hold time. Then, the composition changed to 100% B within 2 min, followed by a 2 min hold time. Finally, the mobile phase was returned to the initial conditions in a 2 min linear gradient. Column temperature was 20 °C. L-Phenylalanine was monitored at 215 nm.

Enzymatic hydrolysis

Pretreated Corn Stover (PCS) or Pretreated Sugarcane Straw (PSCS) were prepared by steam explosion with diluted sulfuric acid at the Abengoa Bioenergy Biomass Pilot Plant in York (Iowa, USA), following the procedure described by Alcántara *et al.* (2016). Hydrolysis of PCS or PSCS (10 g l^{-1}) was performed in 100 mL borosilicate conical flasks containing 1xM9 with pH adjusted to 5.5. The enzymatic cocktail Viscozyme (Sigma-Merck) was added as indicated in Table S3. Glucan content had been previously determined according to the standard biomass analytical procedures by NREL (Rocha-Martín *et al.*, 2018). Samples were incubated in an orbital incubator at 50 °C with shaking at 70 rpm for 24 h.

Scanning Electron Microscopy

Samples were fixed in a 2.5% (v/v) glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.4) for 24 h at 4 °C. Samples were washed in the same buffer (3 times for 15 min, at 4 °C). Post-fixation was performed with 1% (w/v) osmium tetroxide in the dark for 1 h at room temperature. Samples were then washed 3 times for 5 min with distilled water. They were then dehydrated in a gradient of increasing ethanol concentrations from 50% to 100% ethanol and submitted to Critical Point (Anderson, 1951) with carbon dioxide in a Leica EM CPD300 desiccator. Finally, samples were coated by evaporating them with carbon in an EMITECH K975X Carbon evaporator and examined in a high-resolution scanning electron microscope [HRSEM] Auriga *(fib-fesem)* manufactured by Carl Zeiss.

Acknowledgements

Work in our laboratory was supported by grant RTI2018-094370-B-I00 from the Ministry of Science and

Education and counted with FEDER support. We thank Ben Pakuts for critical reading of the manuscript.

Conflict of interest

None declared.

REFERENCES

Abril, M.A., Michán, C., Timmis, K.N., and Ramos, J.L. (1989) Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. *J Bacteriol* **171:** 6782–6790.

Alcántara, M.A.B., Dobruchowska, J., Azadi, P., García, B.D., Molina-Heredia, F.P., and Reyes-Sosa, F.M. (2016) Recalcitrant carbohydrates after enzymatic hydrolysis of pretreated lignocellulosic biomass. *Biotechnol Biofuels* **9:** 1–10.

Álvarez, C., Reyes-Sosa, F.M., and Díez, B. (2016) Enzymatic hydrolysis of biomass from wood. *Microb Biotechnol* **9:** 149–156.

Amore, R., Wilhelm, M., and Hollenberg, C.P. (1989) The fermentation of xylose an analysis of expression of *Bacillus* and *Actinoplanes* xylose isomerase genes in yeast. *Appl Microbiol Biotechnol* **30:** 351–357.

Anderson, T.F. (1951) Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope. *Trans New York Acad Sci* **13:** 130–133.

Aparicio, T., de Lorenzo, V., and Martínez-García, E. (2015) Broadening the SEVA plasmid repertoire to facilitate genomic editing of Gram-negative bacteria. In *Hydrocarbon and Lipid Microbiology Protocols, Springer Protocols Handbooks*. McGenity, T.J., Timmis, K.N., and Fernandez, B.N. (eds.). Berlin, Heidelberg: Springer, pp. 9–27.

Aristodou, A., and Penttilä, M. (2000) Metabolic engineering applications to renewable resource utilization. *Curr Opin Biotechnol* **11:** 187–198.

Bator, I., Wittgens, A., Rosenau, F., Tiso, T., and Blank, L.M. (2020) Comparison of three xylose pathways in *Pseudomonas putida* KT2440 for the synthesis of valuable products. *Front Bioeng Biotechnol* **7:** 480.

Beckham, G.T., Johnson, C.W., Karp, E.M., Salvachúa, D., and Vardon, D.R. (2016) Opportunities and challenges in biological lignin valorization. *Curr Opin Biotechnol* **42:** 40–53.

Calero, P., and Nikel, P. (2019) Chasing bacterial chassis for metabolic engineering: a perspective review from classical to non-traditional microorganisms. *Microb Biotechnol* **12:** 98–124.

del Castillo, T., Ramos, J.L., Rodríguez-Herva, J.J., Fuhrer, T., Sauer, U., and Duque, E. (2007) Convergent peripheral pathways catalyze initial glucose catabolism in *Pseudomonas putida*: genomic and flux analysis. *J Bacteriol* **189:** 5142–5152.

Chandel, A.K., Garlapati, V., Singh, A.K., Antunes, F.A.F., and da Silva, S.S. (2018) The path forward for lignocellulose biorefineries: Bottlenecks, solutions and perspectives on commercialization. *Biores Biotech* **264:** 370–381.

Chen, T., Liu, W.X., Fu, J., Zhang, B., and Tang, Y.J. (2013) Engineering *Bacillus subtilis* for acetoin production from glucose and xylose mixtures. *J Biotechnol* **168:** 499–505.

Daddaoua, A., Krell, T., and Ramos, J.L. (2009) Regulation of glucose metabolism in *Pseudomonas*: The phosphorylative branch and Entner-Doudoroff enzymes are regulated by repressor containing a sugar isomerase domain. *J Biol Chem* **284:** 21360–21368.

Daniels, C., Godoy, P., Duque, E., Molina-Henares, M.A., de la Torre, Jesús, del Arco, J.M., *et al*. (2010) Global regulation of food supply by *Pseudomonas putida* DOT-T1E. *J Bact* **192:** 2169–2181.

Duque, E., and Ramos, J.L. (2019) Twenty-first-century chemical odyssey: fuels versus commodities and cell factories versus chemical plants. *Microb Biotech* **12:** 200–209.

Dvorák, P., and de Lorenzo, V. (2018) Refactoring the upper sugar metabolism of *Pseudomonas putida* for co-utilization of cellobiose, xylose, and glucose. *Metabolic Engin* **48:** 94–108.

Elmore, J.R., Dexter, G.N., Salvachúa, D., O'Brien, M., Klingeman, D.M., Gorday, K., *et al*. (2020) Engineered *Pseudomonas putida* simultaneously catabolizes five major components of corn stover lignocellulose: Glucose, xylose, arabinose, *p*-coumaric acid, and acetic acid. *Metab Eng* **62:** 62–71.

Espeso, D.R., Dvorak, P., Aparicio, T., and de Lorenzo, V. (2021) An automated DIY framework for experimental evolution of *Pseudomonas putida*. *Microb Biotech*. https://doi.org/10.1111/1751-7915.13678

Fellay, R., Frey, J., and Krisch, H. (1987) Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of gram-negative bacteria. *Gene* **52:** 147–154.

Frank, F. (2010) *Current Uses of Synthetic bioloGy*. Biotechnology Industry Organization (BIO). URL www.bio.org/articles/current-uses-synthetic-biology

García-Sanchez, R., Karhumaa, K., Fonseca, C., Sanchez-Nogue, K., Almeida, J.R.M., Larsson, C.U., et al. (2010) Improved xylose and arabinose utilization by industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. *Biotech Biofuel* **3:** 13.

Hahn-Hägerdal, B., and Pamment, N. (2004) Microbial pentose metabolism. *Appl Biochem Biotechnol* **113:** 120.

Hanahan, D. (1985) Techniques for transformation of *E. coli*. In *DNA Cloning: A Practical Approach*. Glover, D.M. (ed). Oxford: IRL Press, pp. 109–135.

Heer, D., and Sauer, U. (2008) Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain. *Microb Biotechnol* **1:** 497–506.

Herrero, M., de Lorenzo, V., and Timmis, K.N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J Bacteriol* **172:** 6557–6567.

Isikgor, F.H., and Becer, C.R. (2015) Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. *Polym Chem* **6:** 4497–4559.

Kawaguchi, H., Vertés, A., Okino, S., Inui, M., and Yukawa, H. (2006) Engineering of a xylose metabolic pathway in

Corynebacterium glutanicum. *Appl Environ Microbiol* **72:** 3418–3428.

Kovach, M.E., Elzer, P.H., Steven Hill, D., Robertson, G.T., Farris, M.A., Roop, R.M., and Peterson, K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166:** 175–176.

Linger, J.g., Vardon, D.r., Guarnieri, M.t., Karp, E.m., Hunsinger, G.b., Franden, M.a., *et al*. (2014) Lignin valorization through integrated biological funneling and chemical catalysis. *Proc Natl Acad Sci USA* **111:** 12013–12018.

Liu, Y., and Nielsen, J. (2019) Recent trends in metabolic engineering of microbial chemical factories. *Curr Opin Biotechnol* **60:** 188–197.

Liu, Y., Xu, Y., Ding, D., Wen, J., Zhu, B., and Zhang, D. (2018) Genetic engineering of *Escherichia coli* to improve L-phenylalanine production. *BMC, Biotech* **18:** 5. https://doi.org/10.1186/s12896-018-0418-1

Loeschcke, A., and Thies, S. (2015) *Pseudomonas putida*–a versatile host for the production of natural products. *Appl Microbiol Biotechnol* **99:** 6197–6214.

Loeschcke, A., and Thies, S. (2020) Engineering of natural product biosynthesis in *Pseudomonas putida*. *Curr Opin Biotechnol* **65:** 213–224.

McKenna, R., and Nielsen, D.R. (2011) Styrene biosynthesis from glucose by engineered *Escherichia coli*. *Metabolic Eng* **13:** 544–554.

Meijnen, J.P., de Winde, J.H., and Ruijssenaars, H.J. (2008) Engineering *Pseudomonas putida* S12 for efficient utilization of D-xylose and L-arabinose. *Appl Environ Microbiol* **74:** 5031–5037.

Meijnen, J.P., de Winde, J.H., and Ruijssenaars, H.J. (2009) Establishment of oxidative D-xylose metabolism in *Pseudomonas putida* S12. *Appl Environ Microbiol* **75:** 2784–2791.

Le Meur, S., Zinn, M., Egli, T., Thony-Meyer, L., and Ren, Q. (2012) Production of medium-chain-length polyhydroxyalkanoates by sequential feeding of xylose and octanoic acid in engineered *Pseudomonas putida* KT2440. *BMC Biotechnol* **12:** 53.

Mishra, P., and Singh, A. (1993) Microbial pentose utilization. *Adv Appl Microbiol* **39:** 91–152.

Molina-Santiago, C., Cordero, B.F., Daddaoua, A., Udaondo, Z., Manzano, J., Valdivia, M., *et al*. (2016) *Pseudomonas putida* as a platform for the synthesis of aromatic compounds. *Microbiology* **162:** 1535–1543.

Nelson, K.e., Weinel, C., Paulsen, I.t., Dodson, R.j., Hilbert, H., Martins dos Santos, V.a.p., *et al*. (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Env Microbiol* **4:** 799–808.

Öhgren, K., Bura, R., Saddler, J., and Zacchi, G. (2007) Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. *Bioresource Technol* **98:** 2503–2510.

Ragauskas, A.j., Beckham, G.t., Biddy, M.j., Chandra, R., Chen, F., Davis, M.f., *et al*. (2014) Lignin valorization: improving lignin processing in the biorefinery. *Science* **344:** 1246843. https://doi.org/10.1126/science.1246843

Ragauskas, A.J., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, C.A., *et al*. (2006) The path forward for biofuels and biomaterials. *Science* **311:** 484–489.

Ramos, J.L., Cuenca, M.S., Molina-Santiago, C., Segura, A., Duque, E., Gómez-García, M.R., *et al*. (2015) Mechanisms of solvent resistance mediated by interplay of cellular factors in *Pseudomonas putida*. *FEMS Microbiol Rev* **39:** 555–566.

Ramos, J.L., Duque, E., Huertas, M.J., and Haïdour, A. (1995) Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. *J Bacteriol* **177:** 3911–3916.

Ramos, J.L., Valdivia, M., García-Lorente, F., and Segura, A. (2016) Benefits and perspectives on the use of biofuels. *Microb Biotechnol* **9:** 436–440.

Rocha-Martín, J., Martínez-Bernal, C., Zamorano, L.S., Reyes-Sosa, F.M., and Díez-García, B. (2018) Inhibition of enzymatic hydrolysis of pretreated corn stover and sugar cane straw by laccases. *Process Biochem* **67:** 88–91.

Rojas, A., Segura, A., Guazzaroni, M.E., Terán, W., Hurtado, A., Gallegos, M.T., and Ramos, J.L. (2003) In vivo and in vitro evidence that TtgV is the specific regulator of the TtgGHI multidrug and solvent efflux pump of *Pseudomonas putida*. *J Bacteriol* **185:** 4755–4763.

Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schwanemann, T., Otto, M., Wierckx, N., and Wynands, B. (2020) *Pseudomonas* as versatile aromatics cell factory. *Biotechnol J* **15:** 1900569.

Segura, A., Molina, L., Fillet, S., Krell, T., Bernal, P., Muñoz-Rojas, J., and Ramos, J.-L. (2012) Solvent tolerance in gram-negative bacteria. *Curr Opin Biotechnol* **23:** 415–421.

Silva-Rocha, R., Martínez-García, E., Calles, B., Chavarría, M., Arce-Rodríguez, A., de las Heras, A., *et al*. (2013) The Standard European Vector Architecture (SEVA): a coherent platform for the analysis and deployment of complex prokaryotic phenotypes. *Nucleic Acids Res* **41:** D666–D675.

Suzuki, T., Hoshino, T., and Matsushika, A. (2019) High-temperature ethanol production by a series of recombinant xylose fermenting *Kluyveromyces marxianus* strains. *Enzyme Microb Technol* **129:** 109359.

Tiso, T., Wierckx, N., and Blank, L.M. (2014) *Non-pathogenic Pseudomona*s as Platform for Industrial Biocatalysis. Singapore: Pan Stanford Publishing.

Tiso, T., Zauter, R., Tulke, H., Leuchtle, B., Li, W.-J., Behrens, B., *et al*. (2017) Designer rhamnolipids by reduction of congener diversity: production and characterization. *Microb Cell Fact* **16:** 225.

Udaondo, Z., Molina, L., Segura, A., Duque, E., and Ramos, J.L. (2016) Analysis of the core genome and pangenome of *Pseudomonas putida*. *Environ Microbiol* **18:** 3268–3283.

Valdivia, M., Galán, J.L., Laffarga, J., and Ramos, J.L. (2016) Biofuels 2020: biorefineries based on lignocellulosic materials. *Microb Biotechnol* **9:** 585–594.

Valdivia, M., Galán, J.L., Laffarga, J., and Ramos, J.L. (2020) A research and technology valuation model for decision analysis in the environmental and renewable

energy sectors. *Renewable Sustain Energy Rev* **122:** 109726.

van Maris, A.J.A., Abbott, D.A., Bellissimi, E., van den Brink, J., Kuyper, M., Luttik, M.A.H., *et al*. (2006) Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. *Antonie van Lecuuwenhock* **90:** 391–418.

Wilhelm, M., and Hollenberg, C.P. (1985) Nucleotide sequence of the *Bacillus subtilis* xylose isomerase gene: extensive homology between the *Bacillus* and *Escherichia coli* enzyme. *Nucleic Acids Res* **13:** 5717–5722.

Wittgens, A., Santiago-Schuebel, B., Henkel, M., Tiso, T., Blank, L.M., Hausmann, R., *et al*. (2018) Heterologous production of long-chain rhamnolipids from *Burkholderia glumae* in *Pseudomonas putida- a step forward to tailor-made rhamnolipids. Appl Microbiol Biotechnol* **102:** 1229–1239.

Wittgens, A., Tiso, T., Arndt, T.T., Wenk, P., Hemmerich, J., Müller, C., *et al*. (2011) Growth independent rhamnolipid production from glucose using the non-pathogenic *Pseudomonas putida* KT2440. *Microb Cell Factor* **10:** 80.

Wynands, B., Lenzen, C., Otto, M., Koch, F., Blank, L.M., and Wierckx, N. (2018) Metabolic engineering of *Pseudomonas taiwanensis* VLB120 with minimal genomic modifications for high-yield phenol production. *Metabol Engin* **47:** 121–133.

Zhang, B.o., Li, X.-l., Fu, J., Li, N., Wang, Z., Tang, Y.-J., and Chen, T. (2016) Production of acetoin through simultaneous utilization of glucose, xylose, and arabinose by engineered *Bacillus subtilis*. *PLoS One* **11(7).e0159298**

Zhang, M., Eddy, C., Deanda, K., Finkelstein, M., and Picataggio, S. (1995) Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis*. *Science* **267:** 240–243.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Southern blot assay to check *P. putida* DOT-T1E and *P. putida* 5PL *gcd* Ω-Km mutant strains. The *gcd* Ω-Km mutant strains are represented with letter "M", control strains (*P. putida* DOT-T1E and *P. putida* 5PL) are represented with letter "C". Probe used for the southern blot assay was the 1009 pb *gcd*-fragment amplified by PCR with T1E_2822Fw and T1E_2822Rv primers, as described in Experimental Procedures

Fig. S2. Growth curves of (A) *P. putida* DOT-T1E and (B) *P. putida* DOT-T1E 5PL and their recombinant strains in minimal medium with 5 g L^{-1} D-glucose. Experiments were carried out as described in Experimental procedures. Blue lines, wild-type strains; red lines, Δ*gcd* mutant strains; green lines, wild-type strains bearing pSEVA633_*xylABE*; purple lines, Δ*gcd* mutant strains bearing pSEVA633_*xylABE*. Curves represent the average of at least two independent assays performed in triplicate. Standard deviations are below 10% of the given values.

Table S1. Xxxxxxxxx.

Table S2. Release of 2G sugars from PSCS by treatment with Viscozyme.

Table S3. Release of 2G sugars from PCS by treatment with Viscozyme.

Table S4. Primers used in this study.

Supplementary Material

