Received: 15 May 2018 | Revised: 20 November 2018 | Accepted: 3 December 2018

DOI: 10.1111/gcbb.12590

ORIGINAL RESEARCH

WILEY GLOBAL CHANGE BIOLOGY BIOENERGY

Growth of engineered *Pseudomonas putida* KT2440 on glucose, xylose, and arabinose: Hemicellulose hydrolysates and their major sugars as sustainable carbon sources

Yan Wang[1*] | **Felix Horlamus**[2*] | **Marius Henkel**[2] | **Filip Kovacic**[3] | **Sandra Schläfle**[4] | **Rudolf Hausmann**[2] | **Andreas Wittgens**[1,5,6] | **Frank Rosenau**[1,5,6]

[1]Institute for Pharmaceutical Biotechnology, Ulm University, Ulm, Germany

[2]Department of Bioprocess Engineering (150k), Institute of Food Science and Biotechnology, University of Hohenheim, Stuttgart, Germany

[3]Institute for Molekular Enzyme Technology (IMET), Heinrich-Heine-University Düsseldorf, Forschungszentum Jülich GmbH, Jülich, Germany

[4]Department of Yeast Genetics and Fermentation Technology (150f), Institute of Food Science and Biotechnology, University of Hohenheim, Stuttgart, Germany

[5]Ulm Center for Peptide Pharmaceuticals (U-PEP), Ulm-University, Ulm, Germany

[6]Department Synthesis of Macromolecules, Max-Planck-Institute for Polymer Research Mainz, Mainz, Germany

Correspondence
Andreas Wittgens, Institute for Pharmaceutical Biotechnology, Ulm University, Ulm, Germany.
Email: andreas.wittgens@uni-ulm.de

Funding information
EU project Horizon 2020 "AD GUT", Grant/Award Number: 686271; Ministry of Science, Research and the Arts of Baden-Württemberg, Grant/Award Number: 7533-10-5-86 A and 7533-10-5-86 B

Abstract

Lignocellulosic biomass is the most abundant bioresource on earth containing polymers mainly consisting of D-glucose, D-xylose, L-arabinose, and further sugars. In order to establish this alternative feedstock apart from applications in food, we engineered *Pseudomonas putida* KT2440 as microbial biocatalyst for the utilization of xylose and arabinose in addition to glucose as sole carbon sources. The D-xylose-metabolizing strain *P. putida* KT2440_*xylAB* and L-arabinose-metabolizing strain *P. putida* KT2440_*araBAD* were constructed by introducing respective operons from *Escherichia coli*. Surprisingly, we found out that both recombinant strains were able to grow on xylose as well as arabinose with high cell densities and growth rates comparable to glucose. In addition, the growth characteristics on various mixtures of glucose, xylose, and arabinose were investigated, which demonstrated the efficient co-utilization of hexose and pentose sugars. Finally, the possibility of using lignocellulose hydrolysate as substrate for the two recombinant strains was verified. The recombinant *P. putida* KT2440 strains presented here as flexible microbial biocatalysts to convert lignocellulosic sugars will undoubtedly contribute to the economic feasibility of the production of valuable compounds derived from renewable feedstock.

KEYWORDS

biocatalyst, D-xylose, hemicellulose hydrolysate, L-arabinose, metabolic engineering, *Pseudomonas putida* KT2440

*Contributed equally.

1 | INTRODUCTION

The development of alternative feedstocks as carbon sources for the industrial biotechnology is one of the major goals to achieve cost-effective and economically efficient bioprocesses, since the price for raw materials especially those of the carbon sources represents a significant proportion of total production costs. Due to insufficient global food supply, the use of feedstocks, which can primarily be used also for food production, is at least ethically questionable and not a preferable basis for the establishment of a truly sustainable bioeconomy. Nevertheless, numerous current biotechnological production processes mostly depend on glucose as carbon source (Wendisch et al., 2016). The conflict between food and biotechnology and the resulting demand to create ethically less problematic processes, which also offer a promising potential for increasing positive socio-economical perception and acceptance by customers of biotechnological products, alternative carbon sources like lignocellulosic biomass, have moved into the focus of attention as renewable and thus sustainable raw materials with a considerable economic potential for industrial biotechnology. An obvious advantage is the fact that they can be recovered from forestry and agro-industrial waste or agricultural residuals (Anwar, Gulfraz, & Irshad, 2014; Mussatto & Teixeira, 2010). Lignocellulosic biomass mainly consists of cellulose, hemicellulose, and lignin containing different polymers. D-Glucose is the only component in cellulose while the composition of hemicelluloses highly varies among different bioresources (Himmel et al., 2010; Shahzadi et al., 2014; Taherzadeh & Karimi, 2008). Pentoses like D-xylose and L-arabinose are the predominant sugars in hemicelluloses and make up to 25% of the total sugar amount in lignocelluloses especially in hardwoods and grasses like wheat, corn, and rice, thereby representing a worldwide available bioresource, but hemicellulose can also contain hexoses like D-glucose, D-mannose, and D-galactose (Brodeur et al., 2011; Kumar, Barrett, Delwiche, & Stroeve, 2009; Lee, 1997). While cellulose is primarily used for other industrial applications, 60 billion tons of hemicelluloses remain almost completely unused every year, which can be hydrolyzed into sugar containing hydrolysates by chemical or enzymatic hydrolysis. This is a prerequisite to use them as substrates for bioprocesses, since typically used microorganisms in industrial biotechnology are naturally unable to use polymers directly (Sun & Cheng, 2002; Xu, Sun, Liu, & Sun, 2006). However, these sugars provided in lignocellulosic hydrolysates can potentially be utilized for the growth of microorganisms and can be converted into different valuable products including biochemical compounds, fine chemicals, food additives, and enzymes (Asgher, Ahmad, & Iqbal, 2013; Iqbal & Asgher, 2013). However, the natural limited metabolic flexibility of many industrial-relevant microorganisms for the use of uncommon carbon sources impedes the efficient utilization of pentose sugars (Kim & Gadd, 2009).

Therefore, several approaches have been used to address this challenge by genetic manipulation and metabolic engineering in different bacteria (Aristidou & Penttilä, 2000; Nieves, Panyon, & Wang, 2015). The pentose phosphate pathway (PPP) is the preferred biochemical route for metabolizing xylose and arabinose present in numerous bacteria. Both xylose and arabinose enter the PPP through D-xylulose 5-phosphate as an intermediate (Stincone et al., 2015). For establishing a xylose degrading pathway in foreign species, heterologous expression of *xylA* (xylose isomerase) and *xylB* (xylulokinase) is a suitable strategy to enable growth on xylose as sole carbon source, which has been successfully performed in various bacteria like *Zymomonas mobilis* (Zhang, Eddy, Deanda, Finkelstein, & Picataggio, 1995), *Corynebacterium glutamicum* (Kawaguchi, Verte, Okino, Inui, & Yukawa, 2006), *Bacillus subtilis* (Chen, Liu, Fu, Zhang, & Tang, 2013), and *Pseudomonas putida* (Le Meur, Zinn, Egli, Thöny-Meyer, & Ren, 2012; Meijnen, Winde, & Ruijssenaars, 2008). Therefore, D-xylose is converted to D-xylulose 5-phosphate through D-xylulose (Gu et al., 2010; Kawaguchi et al., 2006). For the utilization of L-arabinose, a group of three genes, *araB* (ribulokinase), *araA* (L-arabinose isomerase), and *araD* (L-ribulose phosphate 4-epimerase), is necessary, which mediates the conversion of L-arabinose though L-ribulose and L-ribulose 5-phosphate to D-xylulose 5-phosphate (Deanda, Zhang, Eddy, & Picataggio, 1996; Xiong, Wang, & Chen, 2016). This *araBAD* operon has been successfully integrated and heterologously expressed in *C. glutamicum* (Kawaguchi, Sasaki, Vertès, Inui, & Yukawa, 2008) to enable its growth on L-arabinose.

In this present study, we chose *P. putida* KT2440 as a host for generating optimized expression strains by heterologous expression of the *xylAB* and *araBAD* operons to enlarge the available substrate spectrum for this remarkable platform organism. *P. putida* KT2440 has developed into an excellent and robust workhorse for the expression of heterologous genes (Loeschcke & Thies, 2015; Martins Dos Santos, Heim, Moore, Strätz, & Timmis, 2004), possesses an outstanding tolerance toward numerous organic compounds and has been extensively studied for the biosynthesis of biotechnological relevant products, for example, rhamnolipids (Cha, Lee, Kim, Kim, & Lee, 2008; Tiso et al., 2016, 2018; Wittgens et al., 2017, 2018, 2011). Its genome has been completely sequenced, which provides complete insights into its metabolic potential (Nelson et al., 2002; Poblete-Castro, Becker, Dohnt, Santos, & Wittmann, 2012), and especially in Germany, the strain KT2440 is of great importance, since it is the only *P. putida*, which remained in the biosafety level 1 (S1) being a key prerequisite for its use in many industrial applications (BVL, 2012). According to a previous study, *P. putida* KT2440 lacks part of the PPP and is unable for utilizing xylose and arabinose,

but carries the *oprB* gene encoding the outer membrane protein D1, which is responsible for the uptake of xylose and arabinose (Henkel et al., 2012). The growth behaviors of engineered *P. putida* KT2440 strains were investigated in detail during cultivation experiments on glucose, xylose, or arabinose as sole carbon sources as well as on mixtures of these sugars and finally real hemicellulose hydrolysates, to investigate the potential of efficiently utilizing of this cost-effective and renewable feedstock.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and culture conditions

Pseudomonas putida KT2440 (Nelson et al., 2002), *Escherichia coli* DH5α (Grant, Jessee, Bloom, & Hanahan, 1990), and *E. coli* K-12 strain MG1655 (Blattner et al., 1997) were routinely cultivated in lysogenic broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 120 rpm orbital shaking and 30°C for *P. putida* and 37°C for *E. coli*, respectively. Growth experiments using wild-type and engineered *P. putida* strains were carried out in 250-ml baffled Erlenmeyer flasks filled with 25 ml of adapted Wilms-KPi medium (Wilms et al., 2001) containing 13.15 g/L K_2HPO_4, 3.28 g/L KH_2PO_4, 10 g/L $(NH_4)_2SO_4$, 1 g/L NH_4Cl, 4 g/L Na_2SO_4, 50 g/L $MgSO_4{\cdot}7H_2O$ supplemented with 3 ml/L of a trace element solution consisting of 0.18 g/L $ZnSO_4{\cdot}7H_2O$, 0.16 g/L $CuSO_4{\cdot}5H_2O$, 0.1 g/L $MnSO_4{\cdot}H_2O$, 13.92 g/L $FeCl_3{\cdot}6H_2O$, 10.05 g/L EDTA, 0.18 g/L $CoCl_2{\cdot}6H_2O$, 0.662 g/L $CaCl_2{\cdot}2H_2O$, and 10 g/L thiamin HCl. A total amount of 10 g/L D-glucose, D-xylose, L-arabinose, or equal mixtures of these sugars were added to the medium as carbon source.

Hydrolysates were obtained from dried and milled wheat straw, which was first treated in a steam explosion process followed by an enzymatic hydrolysis process carried out for 5 days without using any additives (Schläfle, Tervahartiala, Senna, & Kölling-Paternoga, 2017). These wheat straw hydrolysates containing almost exclusively monomers of D-glucose, D-xylose, and L-arabinose were added to the adapted Wilms-KPi medium complying with a total sugar concentration of 10 g/L, and artificial straw hydrolysates were prepared from single sugars imitating this composition.

Pre-cultures were prepared from glycerol stocks using a total volume of 50 µl stock solution in 25 ml LB medium. Main cultures were inoculated to a starting optical density at 600 nm (OD_{600}) of 0.1 using cells harvested by centrifugation for 10 min at 5,000 *g*.

2.2 | Construction of recombinant plasmids

Genomic DNA of *E. coli* strains DH5α and K-12 MG1655 were isolated using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The amplification of the 2.8-kb *xylAB* operon from *E. coli* DH5α and of the 4.3-kb *araBAD* operon from *E. coli* K-12 strain MG1655 was performed by standard PCR using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Frankfurt a. M., Germany) according to the manufacturer's instructions. The DNA sequences of the primers, obtained from Eurofins Genomics (Ebersberg, Germany), were GTGAAATAACATACTCGAGCAACTGAAAGG and CCCACCCGGTCTAGAAGGGGATAA for *xylAB* and CTTTTCTCGAGCCCACCATTC and GGTTTCTCTAGATTGGCTGTGG for *araBAD*, respectively. The two resulting PCR products were hydrolyzed using restriction enzymes *Xho*I and *Xba*I and subsequently ligated using T4 DNA ligase with the pBBR1MCS-2 expression vector (Kovach et al., 1995) hydrolyzed with the same enzymes. All enzymes were used as recommended by the supplier (Thermo Fisher Scientific, St. Leon-Rot, Germany). *E. coli* DH5α cells were transformed with the resulting recombinant plasmids pBBR1MCS-2_*xylAB* and pBBR1MCS-2_*araBAD* using a standard protocol (Hanahan, 1983). Transformation of *P. putida* KT2440 was performed by electroporation after Choi, Kumar, and Schweizer (2006). Agar plates and liquid media were supplemented with 50 µg/ml kanamycin for selection of positive cells. Recombinant *P. putida* KT2440_*xylAB* and *P. putida* KT2440_*araBAD* strains were additionally screened using solid Wilms-KPi medium plates containing and 10 g/L xylose or arabinose after electroporation.

2.3 | Analytical methods

Cell growth was determined densitometrically by measuring the OD_{600} using a spectral photometer. Culture supernatants were analyzed for sugar concentrations after removing the cells by centrifugation for 5 min at 15,000 *g* and 4°C using the D-Glucose Assay Kit, D-Xylose Assay Kit, and L-Arabinose/D-Galactose Assay Kit (Megazyme, Wicklow, Ireland). The formation of xylonate and arabinoate was determined according to Hofmann et al. (2018).

For the analysis of growth, graphs were created with SIGMAPLOT 13.0 (Systat, San Jose, CA, USA), and a logistic equation with four parameters was used to fit the data. Specific growth rate (μ), maximal specific growth rate (μ_{max}), and biomass to substrate yield (Y_{xls}) were calculated according to the derivation of the polynomial fitting. A maximal standard deviation was applied for all the measurements.

3 | RESULTS

3.1 | D-Xylose and L-arabinose as carbon sources for *P. putida* KT2440

The wild-type strain *P. putida* KT2440 is not able to utilize D-xylose and L-arabinose as sole carbon sources according

to its genetic background (Henkel et al., 2012; Nelson et al., 2002). This was confirmed here by the cultivation of *P. putida* KT2440 in minimal medium containing glucose in comparison with growth experiments using xylose or arabinose as sole carbon sources (Table 1). With glucose, *P. putida* KT2440 reached a significant high cell density ($OD_{600} = 12.1$) with a maximal specific growth rate of 0.61 hr^{-1} and a biomass to substrate yield (Y_{xls}) of 0.37 g/g. In contrast, no growth could be detected after cultivation in either xylose or arabinose containing media after 34 hr. However, in this time the xylose concentration decreased by about 33%, indicating a considerable consumption of xylose. In the same time, an increasing amount of xylonate could be detected, which corresponds to the consumed xylose amount (data not shown). In contrast, a similar depletion of arabinose did not occur during the cultivation.

With the intention to provide *P. putida* KT2440 with efficient pathways for the utilization of xylose and arabinose—which, in addition to glucose, represent the most abundant carbohydrates in lignocelluloses—the dedicated operons *xylAB* and *araBAD* of *E. coli*, respectively, were amplified from *E. coli* chromosomal DNA and subsequently cloned into the pBBR1MCS-2 shuttle vector under transcriptional regulation of the plasmid-encoded *lac*-promoter (P^{lac}). Due to the lack of a functional *lac*-operon and especially the absence of the *lac*-inhibitor (LacI) in *P. putida* KT2440, the expression of the operons controlled by P^{lac} occurs constitutively omitting the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). The resulting recombinant plasmids were finally transferred into *P. putida* yielding the two expression strains *P. putida* KT2440_*xylAB* and *P. putida* KT2440_*araBAD*, respectively. A *P. putida* KT2440 strain harboring the pBBR1MCS-2 empty vector served as a control and showed a growth performance similar to the *P. putida* wild type on glucose with an $OD_{600} = 12.6$, a maximal specific growth rate of 0.58 hr^{-1}, and a biomass yield of 0.34 g/g (Table 1). As expected, this strain did not show any detectable growth after cultivation on xylose or arabinose, but the xylose concentration decreased by 21% while the xylonate concentration increased as observed for the wild type.

Next, the recombinant strain *P. putida* KT2440_*xylAB* was cultivated using one of the three sugars each as the sole carbon source (Table 1; Supporting Information Figure S1a). In contrast to the wild-type and the *P. putida* strain containing the empty vector, this strain was able to grow on xylose and reached an OD_{600} of 9.8, what is similar to its growth on glucose ($OD_{600} = 9.4$). The calculated maximal specific growth rate of 0.39 hr^{-1} on xylose was half as much than on glucose (0.98 hr^{-1}), while the biomass yield was in comparable ranges (xylose: 0.30 g/g, glucose: 0.29 g/g).

It has been reported that a recombinant *P. putida* S12 strain engineered for xylose metabolism showed also unspecific activity of XylA and XylB toward L-arabinose (Meijnen et al., 2008). In our experiments, we could confirm this finding for *P. putida* KT2440_*xylAB* as well, since this strain was also able to grow with arabinose as single carbon source (Table 1, Supporting Information Figure S1a) and reached an OD_{600} of 9.0 with $\mu_{max} = 0.65$ hr^{-1} and $Y_{xls} = 0.27$ g/g comparable to glucose and xylose.

The decreasing sugar concentrations during the cultivation revealed that xylose was consumed from 6 to 22 hr similar to its glucose counterpart proving the presence of a functional and efficient xylose utilization pathway in the recombinant *P. putida* KT2440_*xylAB* strain. In contrast, significant depletion of arabinose was observed in later stages starting from 16 hr and rapidly decreasing until 22 hr.

With the aim to evaluate and potentially improve this basic arabinose utilization and to get deeper understanding in the mechanism behind this "cross-reaction" of the hypothetic unspecific XylAB activities, which we suppose to be responsible for this, we constructed and characterized a

TABLE 1 Growth parameters of different *Pseudomonas putida* strains using various carbon sources

	P. putida WT			*P. putida*_pBBR1MCS-2			*P. putida_xylAB*			*P. putida_araBAD*		
	OD_{600}	μ_{max}	Y_{xls}	OD_{600}	μ_{max}	Y_{xls}	OD_{600}	μ_{max}	Y_{xls}	OD_{600}	μ_{max}	Y_{xls}
Glucose	12.1	0.61	0.37	12.6	0.58	0.34	9.4	0.98	0.29	11.5	0.54	0.35
Xylose	0	0	0	0	0	0	9.8	0.39	0.30	6.9	0.40	0.20
Arabinose	0	0	0	0	0	0	9.0	0.65	0.27	8.4	0.66	0.26
Glucose/xylose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.4	0.54	0.26	6.8	0.49	0.21
Glucose/arabinose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.0	0.59	0.24	8.4	0.62	0.26
Xylose/arabinose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.5	0.54	0.26	7.3	0.66	0.25
Glucose/xylose/arabinose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.4	0.72	0.26	5.9	0.45	0.18
Real hydrolysates	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11.3	0.68	0.34	7.4	0.55	0.22
Artificial hydrolysates	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11.5	0.58	0.35	8.6	0.58	0.26

Note. μ_{max}: maximal specific growth rate (hr^{-1}); n.d.: not determined; OD_{600}: optical density at 600 nm; Y_{xls}: biomass to substrate yield (g/g).

P. putida KT2440 strain carrying a heterologous *araBAD* operon from *E. coli* yielding the new expression strain *P. putida* KT2440_*araBAD*. To our knowledge, this is the first study describing the implementation of a dedicated arabinose metabolism pathway. The new strain was cultivated under the same conditions in the presence of glucose, xylose, or arabinose as its counterpart carrying the *xylAB* operon in the experiments described earlier in this chapter (Table 1, Supporting Information Figure S1b). Unexpectedly, this strain was not only able to grow on glucose and arabinose, but also on xylose. This demonstrates that not only XylAB can mediate growth on arabinose but also that a similar cross- or side reaction exists vice versa allowing *P. putida* to grow on D-xylose enabled by the arabinose-dedicated operon. The growth performance of *P. putida* KT2440_*araBAD* on glucose was similar to both strains described earlier with an $OD_{600} = 11.5$, a maximal specific growth rate of 0.54 hr^{-1} and a biomass yield of 0.35 g/g. However, the lag phase was significantly prolonged, especially in the experiment using xylose, but, astonishingly, also with arabinose resulting in a later start of the exponentially growth phase for both strains between 16 and 20 hr after inoculation. Nevertheless, the *P. putida* KT2440_*araBAD* strain reached a higher cell density of 8.4 with $\mu_{max} = 0.66\ hr^{-1}$ and $Y_{xls} = 0.26$ g/g on L-arabinose compared to the cultivation on xylose ($OD_{600} = 6.9$, $\mu_{max} = 0.40\ hr^{-1}$, $Y_{xls} = 0.20$ g/g) demonstrating a better performance of *P. putida* KT2440_*araBAD* on its expected "preferred" substrate L-arabinose compared to D-xylose.

In a further experiment, the strains *P. putida* KT2440_*xylAB* and *P. putida* KT2440_*araBAD* were cultivated without selective pressure toward plasmid maintenance and thereby cured from their plasmids pBBR1MCS-2_*xylAB* and pBBR1MCS-2_*araBAD*, respectively. This procedure resulted in a complete loss of their ability to utilize xylose and arabinose (data not shown).

In conclusion and without any further attempts toward strain optimization, these results already suggest *P. putida* KT2440_*xylAB* to be a suitable expression strain of choice for the efficient utilization of glucose, xylose, and arabinose.

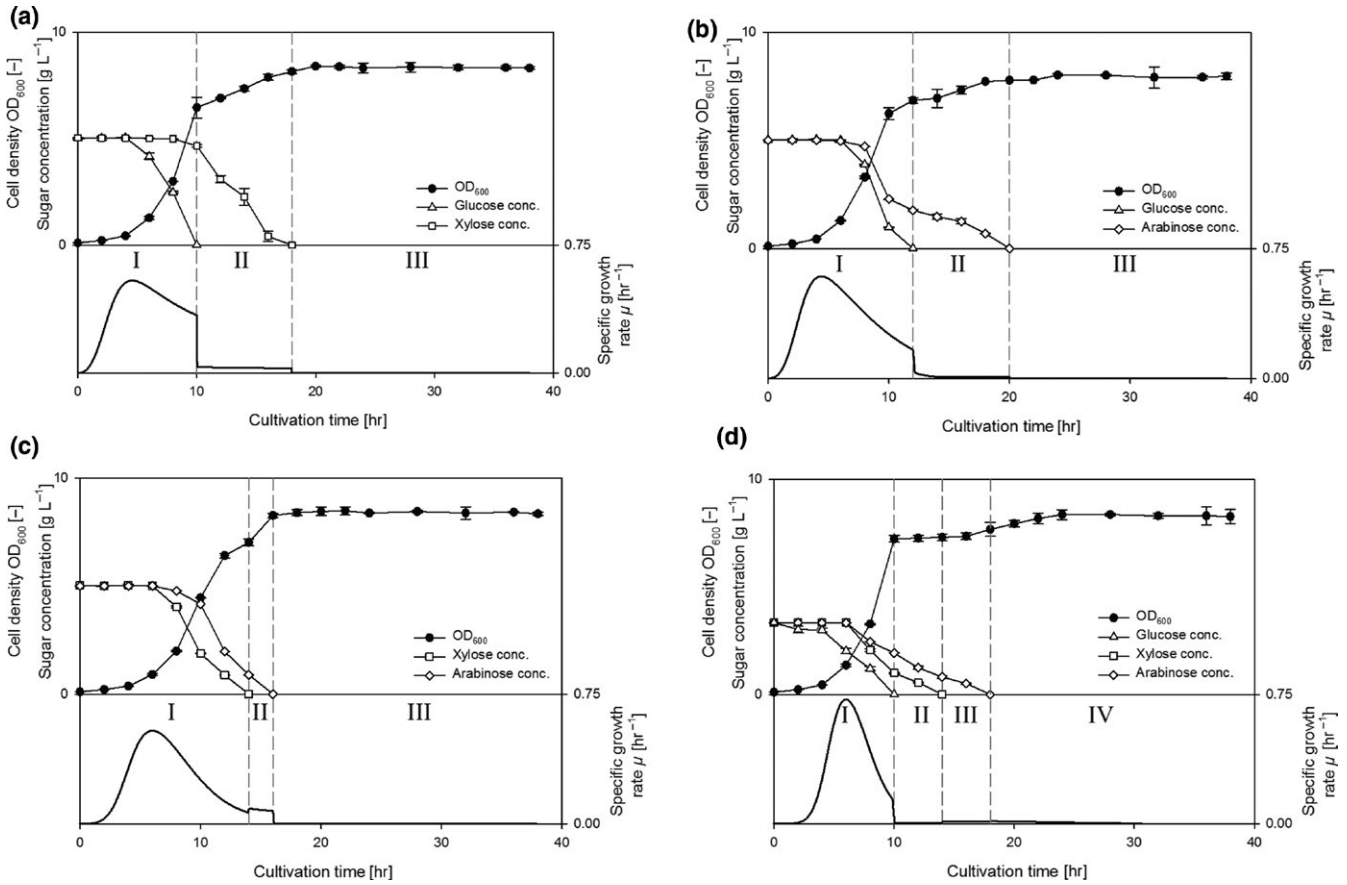

FIGURE 1 Growth performance of *Pseudomonas putida_xylAB* using mixtures of sugars as carbon source. The strain *P. putida* KT2440_*xylAB* (filled circles) was cultivated in Wilms-KPi medium containing mixtures of sugars with a total amount of 10 g/L equally distributed on glucose/xylose (a), glucose/arabinose (b), xylose/arabinose (c), and glucose/xylose/arabinose (d). The consumptions of glucose (empty triangles), xylose (empty squares), and arabinose (empty diamonds) are presented. The specific growth rates of each experiment are separately shown in the lower diagrams, and single growth phases are indicated with Roman numbers. The data points are averages of the results of duplicate measurements. The error bars represent the standard deviations

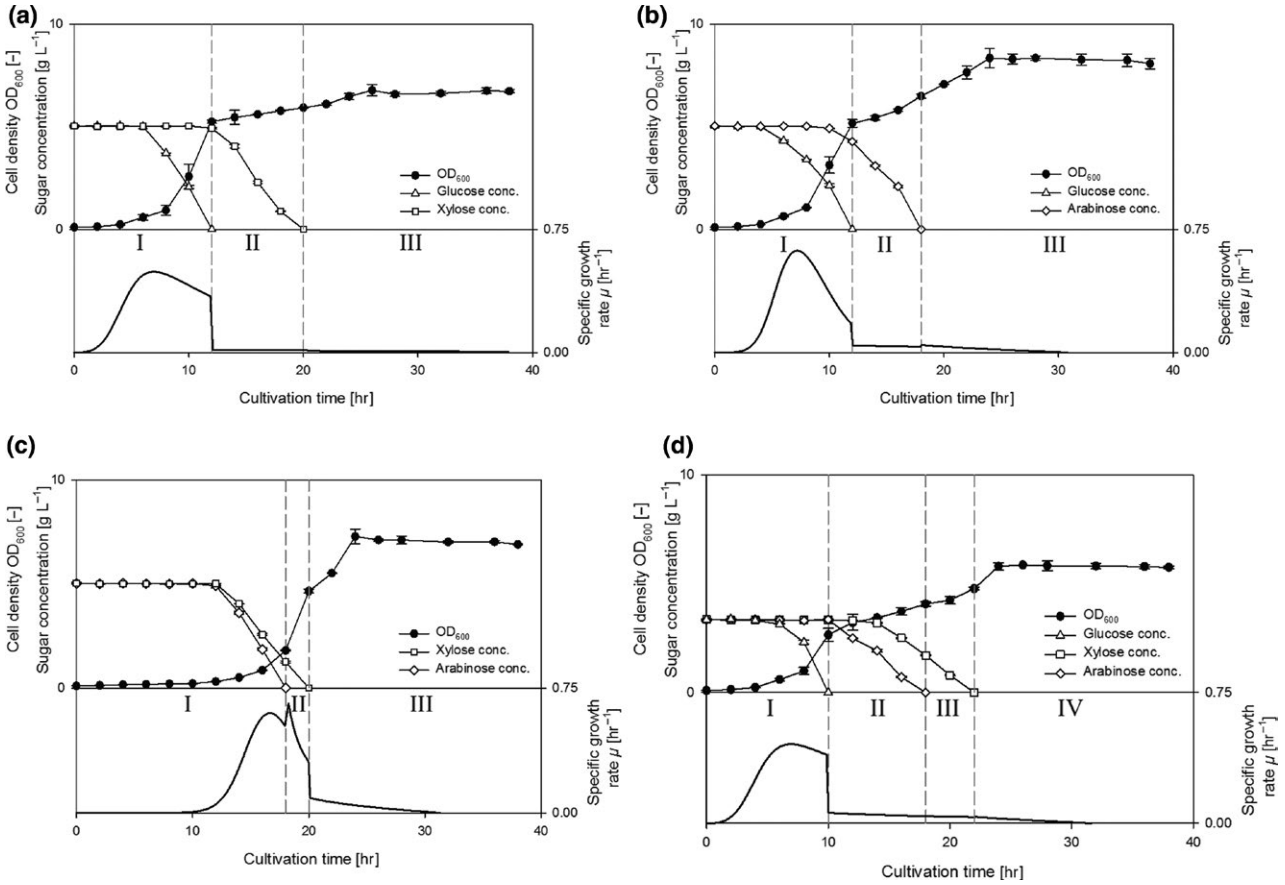

FIGURE 2 Growth performance of *Pseudomonas putida_araBAD* using mixtures of sugars as carbon source. The strain *P. putida* KT2440_*araBAD* (filled circles) was cultivated in Wilms-KPi medium containing mixtures of sugars with a total amount of 10 g/L equally distributed on glucose/xylose (a), glucose/arabinose (b), xylose/arabinose (c), and glucose/xylose/arabinose (d). The consumptions of glucose (empty triangles), xylose (empty squares), and arabinose (empty diamonds) are presented. The specific growth rates of each experiment are separately shown in the lower diagrams, and single growth phases are indicated with Roman numbers. The data points are averages of the results of duplicate measurements. The error bars represent the standard deviations

3.2 | Growth characteristics of recombinant *P. putida* strains on mixtures of sugars

After the verification and characterization of an effective growth of recombinant *P. putida* strains expressing either *xylAB* or *araBAD* with the single sugars glucose, xylose, or arabinose as sole carbon sources, the growth performance on mixtures of two or three of these sugars was investigated.

Both strains showed efficient growth on all sugar mixtures with cell densities and maximal specific growth rates comparable to those on the single sugars. The strain *P. putida* KT2440_*xylAB* reached the following similar values on all different sugar compositions: glucose/xylose: $OD_{600} = 8.4$, $\mu_{max} = 0.54\ hr^{-1}$, $Y_{xls} = 0.26$ g/g; glucose/arabinose $OD_{600} = 8.0$, $\mu_{max} = 0.59\ hr^{-1}$, $Y_{xls} = 0.24$ g/g; xylose/arabinose $OD_{600} = 8.5$, $\mu_{max} = 0.54\ hr^{-1}$, $Y_{xls} = 0.26$ g/g; and glucose/xylose/arabinose $OD_{600} = 8.4$, $\mu_{max} = 0.72\ hr^{-1}$, $Y_{xls} = 0.26$ g/g (Figure 1, Table 1). This performance again revealed the efficient growth of *P. putida* KT2440_*xylAB* on all these three sugars. In contrast, *P. putida* KT2440_*araBAD* exhibited different growth performance on the various sugar combinations (Figure 2, Table 1). Its growth on glucose/xylose ($OD_{600} = 6.8$, $\mu_{max} = 0.49\ hr^{-1}$, $Y_{xls} = 0.21$ g/g) was significantly lower than on glucose/arabinose ($OD_{600} = 8.4$, $\mu_{max} = 0.62\ hr^{-1}$, $Y_{xls} = 0.26$ g/g) confirming a better growth on the supposed specific sugar (L-arabinose). However, the growth on xylose/arabinose ($OD_{600} = 7.3$, $\mu_{max} = 0.66\ hr^{-1}$, $Y_{xls} = 0.25$ g/g) was slightly higher than on glucose/xylose indicating a more efficient utilization when only pentoses were present in the culture media instead of a mixture of hexose and pentose. This hypothesis is supported by the relatively low growth on a mixture of glucose, xylose, and arabinose, where *P. putida* KT2440_*araBAD* reached an OD_{600} of 5.9 and a maximal specific growth rate of $0.45\ hr^{-1}$ and a biomass to substrate yield of 0.18 g/g.

The growth curves for both strains *P. putida* KT2440_*xylAB* and *P. putida* KT2440_*araBAD* on all

sugar mixtures show multiple growth phases (indicated with Roman numbers) along with significant high maximal specific growth rates at the beginning followed by very low ones (Figures 1 and 2, Table 1) based on the switch in metabolic response toward the different carbon sources. Using mixtures of two sugars, the first growth phases abruptly end after 10 hr, when the first sugar is (almost) completely consumed and the growth ceases completely after 20 hr, when also the second sugar was consumed. Exceptions are only the cultivations on xylose/arabinose mixtures where the postponed stops of the first growth phases occurred between 14 and 18 hr followed by reduced second phases. Notably, using glucose/xylose mixtures the utilization of xylose only started after the total consumption of glucose (Figures 1a and 2a, Table 1), while utilization of arabinose already started when the concentration of glucose was decreased, but did not yet reach zero (Figures 1b and 2b, Table 1). When *P. putida* KT2440_*xylAB* and *P. putida* KT2440_*araBAD* were cultivated on the xylose/arabinose mixture, the consumption of both sugars started almost simultaneously, whereby each strain metabolized its specific sugar a bit faster than the second one (Figures 1c and 2c, Table 1). When both strains were cultivated on a mixture composed of all three sugars, first glucose was utilized and completely consumed after 10 hr, followed by xylose and arabinose after 18–22 hr (Figures 1d and 2d, Table 1). Surprisingly, *P. putida* KT2440_*xylAB* started catabolism of xylose and arabinose shortly after metabolizing of glucose began, while in the case of *P. putida* KT2440_*araBAD* the glucose was totally consumed before utilization of both other sugars started. Here again, apart from the consumption of glucose, which is obviously the preferred carbon source, both *P. putida* strains favor its specific pentose sugar and consumed it faster than the remaining one.

In conclusion, both recombinant *P. putida* strains totally consumed all provided sugars, whereby each stain preferred either xylose or arabinose next to glucose as its more specific sugar. However, growth profiles suggest that *P. putida* KT2440_*xylAB* is rather qualified for more effective utilization of these three sugars.

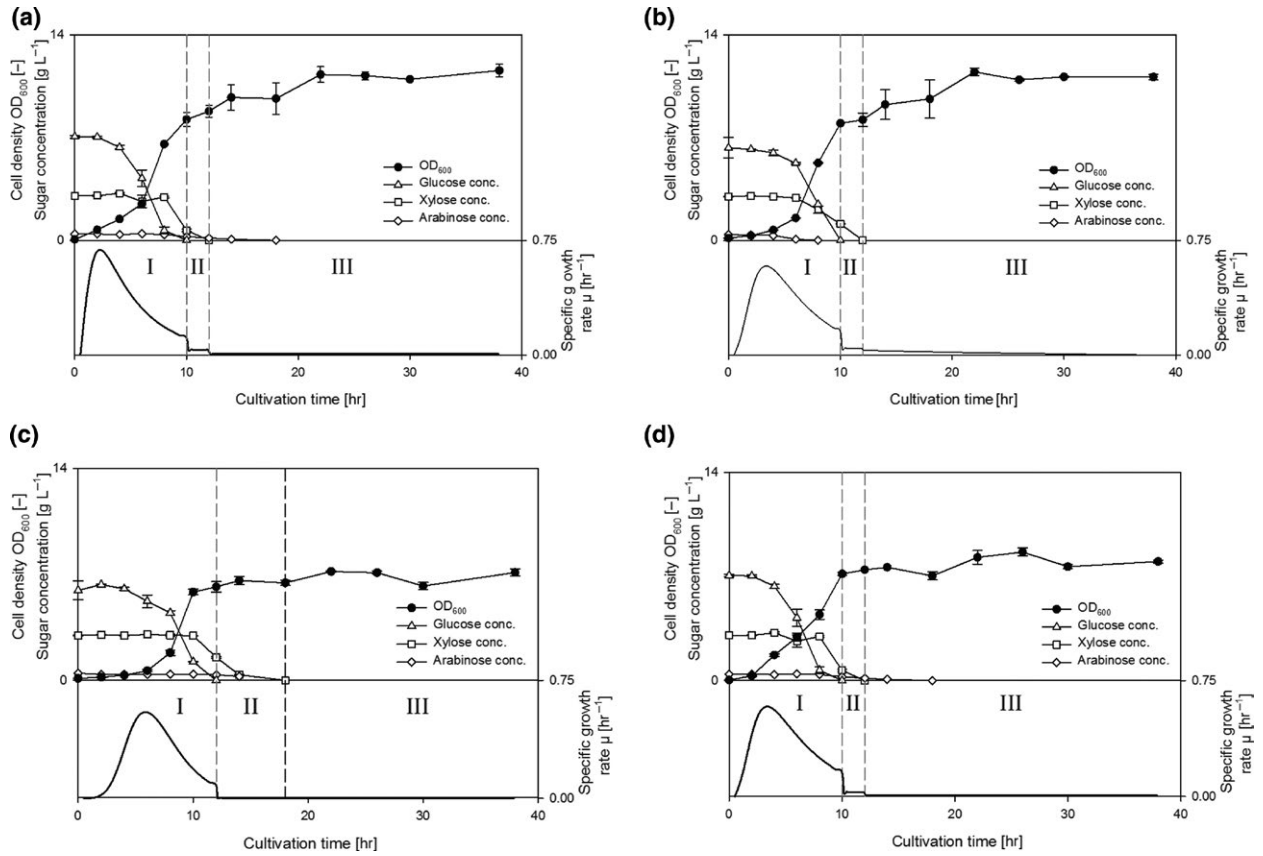

FIGURE 3 Growth performance of recombinant *Pseudomonas putida* using wheat straw hydrolysate as carbon source. The strains *P. putida* KT2440_*xylAB* (a, b) and *P. putida* KT2440_*araBAD* (c, d) (filled circles) were cultivated in Wilms-KPi medium containing real wheat straw hydrolysate (a, c) or artificial hydrolysate with the identical sugar composition (b, d). The growth curves are shown as a smoothed red line. The consumptions of glucose (empty triangles), xylose (empty squares), and arabinose (empty diamonds) are presented. The specific growth rates of each experiment are separately shown in the lower diagrams, and single growth phases are indicated with Roman numbers. The data points are averages of the results of duplicate measurements. The error bars represent the standard deviations

3.3 | Lignocellulosic hydrolysates as substrate for recombinant *P. putida*

Finally, the ability to utilize real lignocellulosic hydrolysates made from wheat straw was tested using the recombinant strains *P. putida* KT2440_*xylAB* and *P. putida* KT2440_*araBAD*. Artificial hydrolysates containing only glucose, xylose, and arabinose were used in comparison with identical concentrations as the real model mixture (Figure 3, Table 1). Both strains were capable to completely utilize the provided glucose, xylose, and arabinose sugars. The real lignocellulosic hydrolysates also contain minimal amounts of additional sugars like mannose or galactose, which are negligible for this present approach. *P. putida* KT2440_*xylAB* reached nearly identical maximal optical densities of 11.3 and 11.5 (Figure 3a,b, Table 1) on the two substrates in comparison with an OD_{600} of 7.4 and 8.6 of *P. putida* KT2440_*araBAD* (Figure 3c,d, Table 1). However, both strains reached comparable maximal specific growth rates when cultivated with real hydrolysates instead of pure sugar mixtures. Surprisingly, the shift to exponential growth of *P. putida* KT2440_*araBAD* on the real hydrolysate takes longer than in all other cases once more underlining *P. putida* KT2440_*xylAB* as a highly suitable strain for efficient utilization of lignocellulosic hydrolysates and its containing sugars.

4 | DISCUSSION

The *P. putida* KT2440 wild type and the strain harboring the empty vector were unable to grow with D-xylose or L-arabinose as sole carbon source. However, a decreasing concentration of xylose could be observed during the cultivation, which is most likely caused by the activity of glucose dehydrogenase (Gcd). Gcd oxidizes xylose to xylonate, which is a dead-end product in the metabolism of *P. putida* and cannot be utilized further (Hardy, Teixeira De Mattos, & Neijssel, 1993; Meijnen et al., 2008). In contrast, no depletion of arabinose was determined indicating the absence of a respective specific enzyme activity and/or a profound incompatibility of arabinose with the specificity of Gcd.

After transformation of *P. putida* KT2440 with *xylAB* or *araBAD* harboring plasmids, the recombinant strains were immediately able to grow on xylose and arabinose without implementation of an adaptation process. In *E. coli,* specific transporters are responsible for the uptake of xylose and arabinose encoded by *xylE* and *araE* (xylose/arabinose:H^+ symporters) or *xylFGH* and *araFGH* (xylose/arabinose ABC transporters). *P. putida* do not encode homologous genes, but possess at least the outer membrane protein D1 (*oprB*) for the transport through the other membrane. According to Meijnen et al. (2008), the sugar transport does not influence its effective utilization indicating an existing unspecific transport mechanism for these sugars into the cytoplasm. However, the growth curves for these experiments show extended lag phases in comparison with the cultivation with glucose indicating the need for a comprehensive metabolic switch to utilize these unusual sugars, possibly depending on regulatory mechanism or slower metabolic fluxes especially in the pentose phosphate pathway. It was earlier reported that successful utilization of xylose after expression of *xylAB* in *P. putida* strain S12 needed a considerably laborious so-called "laboratory evolution" over 36 generations to improve the growth rate from 0.01 to 0.35 hr^{-1} (Meijnen et al., 2008), while this procedure is obviously not required for *P. putida* KT2440 confirming the work by Le Meur et al. (2012), where such an unadapted strain reached a maximal specific growth rate of 0.24 hr^{-1}. The higher growth rate in this present experiment ($\mu_{max} = 0.39\ hr^{-1}$) is probably based on different plasmid characteristics of pBBR1MCS in comparison with the pVLT used in earlier experiments, which shows a much lower copy number and contains an IPTG-dependent *tac*-promoter due to a plasmid-encoded LacI instead of the constitutive P^{lac} in this experiment.

The maximum growth rate for *P. putida_xylAB* (Table 1, Supporting Information Figure S1a) was calculated at the time of very low optical densities (around 0.1). Therefore, it should be noted that measurement errors in this region contribute disproportionally to errors in the specific growth rate, which results in an unusually high growth rate of 0.98 hr^{-1}.

In some experiments with mixtures of sugars especially if arabinose was present, the growth curves further increased although the primary carbon source was already consumed (Figures 2 and 3). The probable reason for this additional growth is the formation of by-products like intracellular deposited polyhydroxyalkanoates, whose production is well described and which serves as carbon and energy store (Poirier, Nawrath, & Somerville, 1995), as well as arabinoate, which is produced in significant amounts in cultures grown under similar experimental conditions (data not shown).

Unexpectedly, the strain *P. putida_araBAD* was not only able to grow on glucose and its supposedly preferred sugar L-arabinose, but showed also unspecific activity toward D-xylose. Nevertheless, *P. putida_araBAD* showed a better growth behavior on L-arabinose compared to D-xylose, which reasonably can be considered to depend on the higher specificity of the Ara-enzymes toward the sugars they are expected to be specific for. A similar cross- or side reaction was already reported for XylA and XylB toward L-arabinose (Meijnen et al., 2008), which was confirmed by our findings. Possibly, in *P. putida* KT2440 the flow of L-arabinose into the pentose phosphate pathway follows an alternative route involving one or more putative enzymes for the conversion of L-ribulose into D-xylulose, which will subsequently be phosphorylated by XylB. However, the exact metabolic route for utilization of L-arabinose by *P. putida* KT2440_*xylAB* needs to be elucidated.

The fact that plasmid curing restored the wild-type phenotype proved the dependency of the results on the presence of the *xylAB* and *araBAD* operons and that these were essential for both, the growth on and the consumption of xylose and arabinose and thereby excluded the possibility that the observed effects were based on mutations in the genome rather than on the plasmid-encoded metabolic enzymes.

The specificity of heterologous proteins certainly explains that the *xylAB*-expressing strain prefers xylose instead of arabinose and the other way around for *P. putida* KT2440_*araBAD*, but this is likely not the reason for different growth profiles of these strains. In *E. coli,* the expression of the *xylAB* and *araBAD* is transcriptionally regulated by transcription factors of the AraC/XylS family, which are widely distributed in gammaproteobacteria (Gallegos, Schleif, Bairoch, Hofmann, & Ramos, 1997). In this study, both operons are controlled by a *lac*-promoter generally uncoupled from native regulatory mechanisms, but conceivably a homologous transcription factor in *P. putida* is able to bind and regulate the heterologous operons. Furthermore, the catabolism of the various sugars used in this study occurs via different metabolic pathways, whose interactions could be responsible for differences in the growth behaviors. The hexose glucose is metabolized through the Entner–Doudoroff pathway, and pentoses like xylose and arabinose are metabolized via the pentose phosphate pathway. The switch between these two metabolic pathways depending on the currently available carbon source could be one reason for the growth curves shapes. In addition, Meijnen, Winde, and Ruijssenaars (2012) report about extensive changes in expression levels in genes involved in both pathways, which are possibly responsible for differences in the utilization of xylose and arabinose.

In this present study, we used wheat straw hydrolysate as a model to demonstrate the potential usage of different lignocellulosic hydrolysates for the growth of recombinant *P. putida* KT2440. Moreover, a wide variety of raw materials including other grain straws, grasses, sugarcane bagasse, miscanthus as well as hard and soft woods are potential bioresources, which can be used as carbon sources after pretreatment and hydrolysis (Hahn-Hägerdal, Galbe, Gorwa-Grauslund, Lidén, & Zacchi, 2006; Lavarack, Griffin, & Rodman, 2002; Mussatto & Teixeira, 2010; Saha, 2003). In future, large-scale processes will be developed for the production of valuable products based on lignocellulosic biomass, to move on from research level to commercial applications.

ACKNOWLEDGEMENTS

This work by Wang et al. was supported by a grant from the Ministry of Science, Research and the Arts of Baden-Württemberg (MWK) Az: 7533-10-5-86 A and 7533-10-5-86 B. Furthermore, the authors acknowledge generous support by the bioeconomy graduate program BBW ForWerts, supported by the MWK and the EU project Horizon 2020 "AD GUT" No. 686271.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ORCID

Marius Henkel https://orcid.org/0000-0002-5343-9661
Andreas Wittgens https://orcid.org/0000-0001-6771-4380

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SUPPORTING INFORMATION

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

How to cite this article: Wang Y, Horlamus F, Henkel M, et al. Growth of engineered *Pseudomonas putida* KT2440 on glucose, xylose, and arabinose: Hemicellulose hydrolysates and their major sugars as sustainable carbon sources. *GCB Bioenergy*. 2019;11:249–259. https://doi.org/10.1111/gcbb.12590