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Potential of biotechnological conversion of lignocellulose hydrolyzates by *Pseudomonas putida* KT2440 as a model organism for a bio-based economy

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Abstract

Lignocellulose-derived hydrolyzates typically display a high degree of variation depending on applied biomass source material as well as process conditions. Consequently, this typically results in variable composition such as different sugar concentrations as well as degree and the presence of inhibitors formed during hydrolysis. These key obstacles commonly limit its efficient use as a carbon source for biotechnological conversion. The gram-negative soil bacterium *Pseudomonas putida* KT2440 is a promising candidate for a future lignocellulose-based biotechnology process due to its robustness and versatile metabolism. Recently, *P. putida* KT2440_*xylAB* which was able to metabolize the hemicellulose (HC) sugars, xylose and arabinose, was developed and characterized. Building on this, the intent of the study was to evaluate different lignocellulose hydrolyzates as platform substrates for *P. putida* KT2440 as a model organism for a bio-based economy. Firstly, hydrolyzates of different origins were evaluated as potential carbon sources by cultivation experiments and determination of cell growth and sugar consumption. Secondly, the content of major toxic substances in cellulose and HC hydrolyzates was determined and their inhibitory effect on bacterial growth was characterized. Thirdly, fed-batch bioreactor cultivations with hydrolyzate as the carbon source were characterized and a diauxic-like growth behavior with regard to different sugars was revealed. In this context, a feeding strategy to overcome the diauxic-like growth behavior preventing accumulation of sugars is proposed and presented. Results obtained in this study represent a first step and proof-of-concept toward establishing lignocellulose hydrolyzates as platform substrates for a bio-based economy.

KEYWORDS

bioconversion, bioeconomy, biomass, biorefinery, hemicellulose, hydrolysis, lignocellulose, *Pseudomonas putida* KT2440

Felix Horlamus and Yan Wang contributed equally to this work.

1 | INTRODUCTION

Lignocellulose is a potential key carbon resource for a future bio-based economy as it is the most abundant renewable raw material on earth. Furthermore, it is not a direct competitor to food production as it accumulates in large quantities as waste in the wood, food, and agricultural industry (Anwar, Gulfraz, & Irshad, 2014; Jørgensen, Kristensen, & Felby, 2007; Lange, 2007; Naik, Goud, Rout, & Dalai, 2010; van Dyk, Gama, Morrison, Swart, & Pletschke, 2013). Lignocellulose is the structural framework of woody plant cell walls and consists mainly of lignin, cellulose (CE), and hemicellulose (HC). The amorphous heteropolymer lignin, consisting of phenylpropane units, is mainly interesting for material sciences and as a source for aromatic polymers (Upton & Kasko, 2016). CE consists of glucose linked by β-1,4 glucosidic bonds and most microorganisms are able to metabolize its depolymerization product glucose. On the other hand, the HC fraction remains mostly unused although approximately 60 billion tons of HCs are produced annually (Gatenholm & Tenkanen, 2003; Shahzadi et al., 2014; Wyman, 1994; Xu, Sun, Liu, & Sun, 2006). HCs are a group of heterogeneous polysaccharides consisting of different monomers such as D-xylose, D-mannose, D-arabinose, D-glucose, and sugar acids. In contrast to CE, the structure of HCs differs from plant to plant (Gatenholm & Tenkanen, 2003; Hendriks & Zeeman, 2009; Timell, 1967). In hardwoods like beech (Itoh, Wada, Honda, Kuwahara, & Watanabe, 2003; Lu, Yamauchi, Phaiboonsilpa, & Saka, 2009; Teleman, Tenkanen, Jacobs, & Dahlman, 2002) and grasses (*Poaceae*), such as miscanthus (Schläfle, Tervahartiala, Senn, & Kölling-Paternoga, 2017), corn (Jørgensen et al., 2007), or wheat (Jørgensen et al., 2007; Schläfle et al., 2017), xylose is the dominant monosaccharide in HC. In softwood like fir and spruce (Hoyer, Galbe, & Zacchi, 2009; Tengborg et al., 1998), mannose is the predominant monosaccharide component. In order to use lignocellulose as a carbon source for biotechnological processes it usually has to be depolymerized since most industrially used microorganisms are not able to metabolize this compact and complex polymer. A common method is chemically or enzymatically catalyzed hydrolysis (Sun & Cheng, 2002; Taherzadeh & Karimi, 2007a, 2007b; van Dyk & Pletschke, 2012). Hydrolysis is usually preceded by pretreatment such as water steam explosion or organosolv treatment (Alvira, Tomas-Pejo, Ballesteros, & Negro, 2010; Domínguez de María, Grande, & Leitner, 2015; Mosier et al., 2005; Taherzadeh & Karimi, 2008). During these processes, inhibitors are formed, for instance, CE- and HC-derived furan aldehydes and aliphatic acids as well as lignin-derived phenolic compounds. Usually less toxic substances are formed in catalytic processes like hydrolysis than in thermochemical depolymerization processes such as pyrolysis, but nevertheless this may lead to issues when applied in biological systems (Arnold, Moss, Henkel, & Hausmann, 2017; Jönsson, Alriksson, & Nilvebrant, 2013; Palmqvist & Hahn-Hägerdal, 2000). The inhibitor concentrations showed great fluctuation between different hydrolyzates, as summarized by (Chandel, da Silva, & Singh, 2011). Acetic acid concentration varies from 0.4 g/L (Alriksson, Cavka, & Jönsson, 2011) to 5.45 g/L (Chandel & Singh, 2011), furfural concentration from 0.15 g/L (Nigam, 2001) to 2.2 g/L (Qian et al., 2006), and hydroxymethylfurfural (HMF) concentration from 0.07 g/L (Villarreal, Prata, Felipe, & Almeida E Silva, 2006) to 3.3 g/L (Alriksson et al., 2011). This, however, can be countered with an adjusted process technology, for example, milder hydrolysis methods to decrease inhibitor formation or separation techniques to remove inhibitors. However, this either leads to lower yields of fermentable sugar or requires additional process steps. For this reason, a promising solution could be the utilization of robust microorganisms like *Pseudomonas putida* which display a comparatively low sensitivity toward inhibitors (Martins Dos Santos, Heim, Moore, Strätz, & Timmis, 2004; Poblete-Castro, Becker, Dohnt, Dos Santos, & Wittmann, 2012; Roma-Rodrigues, Santos, Benndorf, Rapp, & Sá-Correia, 2010; Santos, Benndorf, & Sá-Correia, 2004; Segura et al., 2005). *P. putida* KT2440 is a plasmid-free derivative of the strain *P. putida* mt-2 isolated in Japan (reviewed by Nakazawa, 2003). This gram-negative, ubiquitous, saprophytic soil bacterium has become a remarkable workhorse for biotechnical processes (Loeschcke & Thies, 2015; Martins Dos Santos et al., 2004). As an example, it is a suitable host for the production of the biosurfactant rhamnolipid (Arnold, Henkel, et al., 2019; Beuker, Barth, et al., 2016, Beuker, Steier, et al., 2016; Cha, Lee, Kim, Kim, & Lee, 2008; Tiso et al., 2017; Wittgens et al., 2011, 2017, 2018). Unlike many other *Pseudomonads*, *P. putida KT2440* is classified as biosafety level 1 according to American Type Culture Collection. Furthermore, the complete and annotated genome sequence for *P. putida KT2440* is available (Nelson et al., 2002). Its versatile metabolism and robustness against numerous organic compounds (Martins Dos Santos et al., 2004; Nelson et al., 2002; Poblete-Castro et al., 2012) makes it a candidate for a next-generation lignocellulose biorefinery strain. However, *P. putida* KT2440 wild type is not able to metabolize the main HC sugars, xylose and arabinose, as it lacks the enzymes xylose isomerase (*xylA*), xylulose kinase (*xylB*), L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), and L-ribulose-5-phosphate 4-epimerase (Henkel et al., 2012). In the recent past, *P. putida* KT2440 has been engineered toward metabolization of HC sugars (Dvořák & de Lorenzo, 2018; Meijnen, Winde, & Ruijssenaars, 2008; Wang et al., 2019). Similarly, in this study, strain *P. putida* KT244_*xylAB* carrying the plasmid pBBR1MCS-2 with the *xylAB* operon from *Escherichia coli* DH5α was used. Upon introduction of the *xylAB* operon, the resulting strain was able to metabolize xylose and arabinose

resulting in similar growth rates compared to glucose (Wang et al., 2019).#AuthorQueryReply##AuthorQueryReply##AuthorQueryReply#

Building on this, the intent of the current study was to evaluate *P. putida* KT2440 as a platform model organism for bioconversion of different lignocellulose hydrolyzates. Firstly, hydrolyzates of different origins were evaluated as potential carbon sources for the developed HC sugar metabolizing *P. putida* by cultivation experiments. Secondly, the content of major toxic substances in CE- and HC hydrolyzates was determined and their inhibitory effect on bacterial growth was characterized. Thirdly, a fed-batch cultivation strategy in a bioreactor with hydrolyzate as the carbon source was proposed. Results obtained in this study represent a first step and proof-of-concept toward establishing lignocellulose hydrolyzates as platform substrates for a bio-based economy.

2 | MATERIALS AND METHODS

2.1 | Chemicals and standards

If not stated otherwise, all chemicals were purchased from Carl Roth GmbH. HMF was obtained from AVA Biochem BSL AG.

2.2 | Hydrolyzates

As potential carbon sources, hydrolyzates from different sources were evaluated. The main differences between each hydrolyzate are related to the manufacturing process, the applied raw material as well as the utilized lignocellulose fractions:

- For hydrolysis with diluted sulfuric acid, CE and HC were hydrolyzed simultaneously with beech (hydrolyzate a) and spruce (hydrolyzate b). Hydrolysis was performed in a fixed bed reactor loaded with biomass in chip size. The reactor was heated up to the reaction temperature of 180°C with a constant water flow. When the reaction temperature was reached, 0.05 mol/L sulfuric acid was introduced to the reactor and the hydrolyzate was constantly removed.
- The two-step acid hydrolysis included a high concentration of hydrochloric acid (32% and 28%), HC fraction, and rice hulls (hydrolyzate c; Green Sugar AG) as described previously (Green Sugar AG, 2018).
- Steam explosion followed by enzymatic hydrolysis included CE/HC, miscanthus (hydrolyzate d), and wheat straw (hydrolyzate e) as described previously (Schläfle et al., 2017).
- For the organosolv process followed by enzymatic hydrolysis, CE fraction (hydrolyzate f), HC fraction (hydrolyzate g), and beech were used as described in Dörsam et al. (2017).

Initially, the pH of the hydrolyzates was measured (SevenCompact, Mettler-Toledo GmbH) and adjusted to 7.0 using a 10 M sodium hydroxide solution. Later, samples were centrifuged (12,000 × g, 20°C, 10 min) with Heraeus Multifuge X3 (Thermo Fisher Scientific GmbH) and subsequently the supernatant was sterile filtered (Rotilabo®-syringe filters, 0.22 µm pore size; Carl Roth GmbH). Finally, the hydrolyzates were concentrated or diluted to a concentration of 100 or 150 g/L.

2.3 | Strains and plasmids

Pseudomonas putida KT2440 wild type (Nelson et al., 2002) was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures listed under strain number 6125. *P. putida* KT2440 pBBR2MCS-2_*xylAB* contains the *xylAB* operon from *E. coli* DH5α (Grant, Jessee, Bloom, & Hanahan, 1990). The operon encodes for the genes *xylA* (xylose isomerase) and *xylB* (xylulose kinase), which are required for metabolizing the HC monosaccharides. The construction of the plasmid is described in detail by Wang et al. (2019).

2.4 | Media

Preculture was performed with lysogenic broth (LB) medium: 5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl; pH 7.0. All other cultivations were carried out with adapted Wilms medium (Wilms et al., 2001) together with an adapted phosphate buffer system (Beuker, Steier, et al., 2016): 500 g/L 100 mM KP_i buffer (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 $MgSO_4 \cdot 7H_2O$), 3 ml/L trace element solution (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 Titriplex III, 0.18 g/L $CoCl_2 \cdot 6H_2O$, 0.662 g/L $CaCl_2 \cdot 2H_2O$, pH 2), and 0.01 g/L thiamin HCl. A total amount of 10 g/L D-(+)-glucose, D-(+)-xylose, L-(+)-arabinose, equal mixtures of these sugars, or lignocellulose hydrolyzates were added to the medium as carbon source. For strains with pBBR2MCS-2 plasmid, kanamycin with a concentration of 50 µg/ml was added to the medium as a selection marker.

The medium was modified for bioreactor cultivations. The batch medium contained a lower sugar concentration of 5 g/L. As a carbon source, wheat straw hydrolyzates were used. The feed medium had a higher sugar concentration of 150 g/L and a higher nitrogen concentration (NH_4Cl: 2.51 g/L, $(NH_4)_2SO_4$: 25.1 g/L).

2.5 | Cultivation (Erlenmeyer flasks)

For the preculture, 25 ml LB medium was inoculated with 100 µl glycerol stock solution. After overnight cultivation, 25 ml

Wilms–KP_i medium were inoculated with an initial optical density of 0.1 at 600 nm (OD_{600}). The cultivations took place in 250-ml Erlenmeyer baffled flasks at 30°C and 120 rpm in an incubation shaker (Innova 44R, Eppendorf AG). For storage, cultures were mixed with glycerol (25% v/v) and frozen at −80°C.

2.6 | Cultivation (bioreactor)

2.6.1 | Equipment

The experiments were performed in a 2 L bench-top bioreactor (Labfors 4; Infors AG). The process control and the recording of the results were carried out with a bioreactor control software IRIS (Infors AG). Temperature was kept constant at 30°C. The pH value was determined with a pH sensor (EasyFerm Bio K8224; Hamilton Company) and was adjusted to 7.0 with 4 M phosphoric acid and 4 M sodium hydroxide solution. Oxygen partial pressure (pO_2) of the medium was measured with an optical probe (VisiFerm DO 225; Hamilton Company) and controlled to 30% by adjusting stirring rate (300–1,250 rpm) and aeration with compressed air (0.1–0.5 vvm). In addition, carbon dioxide and oxygen content of exhaust gas was measured with a gas analyzer (INFORS HT; Infors AG). Feed medium was added with the laboratory peristaltic tube pump (model 323Du/D; Watson-Marlow Fluid Technology Group) and controlled via MATLAB (The MathWorks, Inc.).

2.6.2 | Experimental setup (fed-batch)

Five hundred milliliters of Wilms–KP_i medium was inoculated with a starting OD_{600} of 0.1 and the feed was started when all sugars were consumed as indicated by a rise in pO_2. A specific growth rate μ of 0.44/hr was applied for calculation of the feed rate to allow for exponential growth (Beuker, Barth, et al., 2016). Antifoam agent (Contraspum A4050; Zschimmer & Schwarz GmbH & Co. KG Chemical Factories) was added to the medium in case of excessive foaming as required.

2.7 | Analytical methods

2.7.1 | Cell density

Cell density was determined immediately after sampling. The optical density at 600 nm (OD_{600}) was measured via a cell density meter (CO8000; Biochrome) and samples were diluted as required with saline solution (0.9%).

2.7.2 | Monosaccharides

Samples were centrifuged (5 min, 4°C, 4,700 rpm) and the supernatant was used to measure D-glucose, D-xylose, L-arabinose, and D-mannose content. This was carried out with the following enzyme assays: D-glucose (R-Biopharm AG), D-Xylose Assay Kit, L-Arabinose/D-Galactose Assay Kit, and D-Mannose/L-Fructose/D-Glucose Assay (Megazyme u.c., Co.).

2.7.3 | Formic acid, acetic acid, furfural, HMF

The quantitative analysis of formic acid, acetic acid, furfural, and HMF was performed via high-performance liquid chromatography.

The furan compounds HMF and furfural were separated at 20°C in a Lichrospher 100 RP-18 column (Merck). As the mobile phase, a water–acetonitrile eluent (9:1 v/v) at a flow rate of 1.4 ml/min was used, and a UV detector was operated at 290 nm. Formic acid and acetic acid were analyzed with an Aminex HPX-87H column (Bio-Rad) at a temperature of 25°C. The eluent was 0.004 mol/L sulfuric acid at a flow rate of 0.65 ml/min and detection was performed by refractive index detector and diode array detector.

2.8 | Inhibitory effect of formic acid, acetic acid, furfural, and HMF on the growth of *P. putida* KT2440

Pseudomonas putida KT2440 wild type and *P. putida* KT2440_*xylAB* were cultivated in Wilms–KP_i medium as described above. In addition, formic acid, acetic acid, furfural, and HMF were added in different concentrations to the medium, respectively. The pH value of the medium was adjusted to 7.0 with 10 M sodium hydroxide after addition of the inhibitors.

2.9 | Software for graphical analysis, biological replicates, and measurement error

Creation of graphs and graphical analysis of measured data were performed using scientific graphing and data analysis software (SigmaPlot; Systat Software Inc.). All data, if not stated otherwise, were obtained as duplicates from at least two independent biological experiments, and measurement results are presented as mean ± *SD*.

3 | RESULTS

3.1 | Growth performance of *P. putida* KT2440_*xylAB* with lignocellulose monosaccharides

Pseudomonas putida KT2440_*xylAB* was able to metabolize all main lignocellulose monosaccharides (Wang et al., 2019). Based on these results, an evaluation of different

TABLE 1 Monosaccharide concentration and inhibitory concentration in lignocellulose hydrolyzates of different origin

Hydrolyzate	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Mannose (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Furfural (g/L)	HMF (g/L)
Sulfuric acid, CE/HC, beech (a)	7.5	2.3	0.2	ND	1.8	1.9	2.0	4.8
Sulfuric acid, CE/HC, spruce (b)	7.8	0.4	0.1	1.7	0.1	0.1	0.6	2.4
Hydrochloric acid, HC, rice hulls (c)	2.5	5.9	1.6	ND	ND	ND	0.1	ND
Steam explosion and enzymes, CE/HC, miscanthus (d)	7.5	2.3	0.2	ND	ND	0.5	0.2	ND
Steam explosion and enzymes, CE/HC, wheat straw (e)	6.8	3.0	0.2	ND	ND	0.2	0.2	ND
Organosolv process and enzymes, CE, beech (f)	7.9	2.1	ND	ND	ND	0.3	ND	ND
Organosolv process and enzymes, HC, beech (g)	1.2	8.0	0.8	ND	1.3	2.3	0.1	ND

Note: All hydrolyzates were adjusted to a total monosaccharide concentration of 10 g/L.
Abbreviations: CE, cellulose; HC, hemicellulose; ND, not detectable.

lignocellulose hydrolyzates (Table 1) for use as carbon sources in biotechnological processes with *P. putida* KT2440_*xylAB* was performed (Figure 1; Table 2).

As a reference, cultivation with glucose as a sole carbon source was performed (Figure 1h; Table 2). The cells reached a maximum OD_{600} of 12.7, with a maximal specific growth rate μ_{max} of 0.7/hr and biomass to substrate ratio $Y_{X|S}$ of 0.41 g/g. Glucose was completely consumed. With beech and spruce hydrolyzates treated with sulfuric acid (hydrolyzates a + b), no cell growth and no decrease in the sugar concentration could be measured over the entire cultivation period of 48 hr (Figure 1a,b; Table 2). *P. putida* KT2440_*xylAB* had a similar growth performance with rice hull samples hydrolyzed with hydrochloric acid (hydrolyzate c) as with glucose with a maximum OD_{600} = 11.5, μ_{max} = 0.4/hr and $Y_{X|S}$ = 0.41 (Figure 1c; Table 2). The bacteria showed a diauxic-like growth pattern. First, *P. putida* KT2440_*xylAB* metabolized mainly glucose (phase I) and when glucose was almost consumed the strain metabolized xylose and arabinose (phase II) and at the end only small amounts of arabinose remain in the medium (phase III). Next, hydrolyzates depolymerized via steam explosion and enzymes were applied (Figure 1d,e; Table 2). With miscanthus (hydrolyzate d), maximum OD_{600} = 11.3, μ_{max} = 0.4/hr, and $Y_{X|S}$ = 0.38 was detected. Using wheat straw (hydrolyzate e), slightly higher growth rates were measured (OD_{600} = 11.7, μ_{max} = 0.5/hr and $Y_{X|S}$ = 0.38). In both cases, first glucose (phase I) and then xylose and arabinose were consumed (phase II). The highest values with OD_{600} = 12.6, μ_{max} = 0.7/hr, and $Y_{X|S}$ = 0.42 were obtained with the CE fraction of the organosolv process (figure f, Table 2). The strain metabolized glucose first and then xylose. No growth could be verified applying the HC fraction of the organosolv process (figure g, Table 2).

The growth parameters of all cultivation experiments are summarized in Table 2. As a comparison, cultivations of the wild type and cultivations with glucose as carbon source are presented. As expected, the maximum OD_{600} of *P. putida* KT2440 wild type (12.7) and *P. putida* KT2440_*xylAB* (12.4) were very similar using glucose as carbon source. With the CE fraction of the organosolv process (hydrolyzate f), *P. putida* KT2440_*xylAB* reached a slightly higher maximum OD_{600} of 12.6 as the wild type strain with a maximum OD_{600} of 11.5. Overall, the recombinant strain displayed higher growth rates than the wild-type strain with hydrolyzates containing HC monosaccharides. This was particularly evident with the pure HC samples (hydrolyzate c) as the wild type strain reached a maximum OD_{600} of 4.1 and the *xylAB* strain reached a maximum OD_{600} of 11.5. For better comparability, the relative carbon hydrolyzate conversion index (RCHC) was defined. It is defined as the ratio of biomass of respective cultivation to the cultivation with *P. putida KT2440* wild type and glucose as carbon source. Furthermore, this index was calculated to obtain system-independent data, which facilitates the comparability of other work on carbon sources/hydrolyzates and gene constructs. With *P. putida* KT2440_*xylAB* the highest RCHC was calculated with hydrolyzate f (100%) followed by hydrolyzate e (93%), hydrolyzate c (90%), and hydrolyzate d (90%; Table 2).

3.2 | Inhibitors

The inhibitory effect of major toxic substances in lignocellulose hydrolyzates on the growth of *P. putida* KT2440 was investigated.

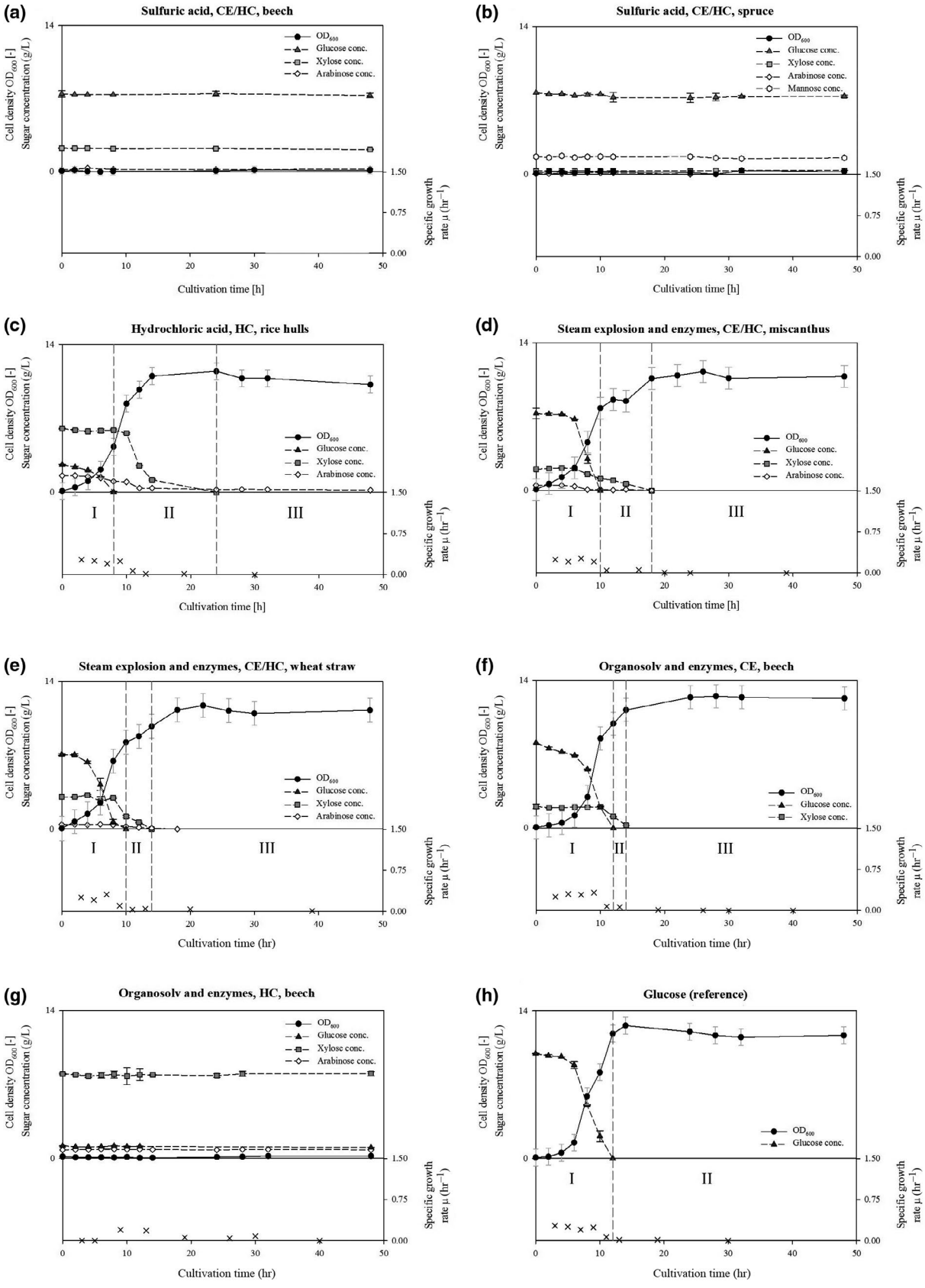

FIGURE 1 Time course of cultivation of *Pseudomonas putida* KT2440_*xylAB* with different lignocellulose hydrolyzates as carbon source. The specific growth rates are separately shown in the lower diagrams and single growth phases are indicated with roman numbers: phase I, mainly consumption of the first sugar (glucose); phase II, mainly consumption of the second (xylose) and third sugar (arabinose); phase III, consumption of the third sugar (arabinose). Subfigures indicated by lowercase letters correspond to the applied hydrolyzate (Table 2). CE, cellulose; HC, hemicellulose

TABLE 2 Growth parameter of cultivations of *Pseudomonas putida* KT2440 with different hydrolyzates as carbon source

Substrate	OD_{max}	Bacterial biomass (g/L)	Max. spec. growth rate μ_{max} (1/hr)	$Y_{X|S}$ (g/g)[a]	RCHC (%)[b]
P. putida KT2440 (wild type)					
Glucose (reference)	12.7 ± 0.4	4.2	0.6	0.42	100
Sulfuric acid; CE/HC; beech (a)	0.1 ± 0.1	—	—	—	—
Sulfuric acid; CE/HC; spruce (b)	0.1 ± 0.0	—	—	—	—
Hydrochloric acid; HC; rice hulls (c)	4.1 ± 0.1	1.4	0.4	0.14	33
Steam explosion and enzymes; CE/HC, miscanthus (d)	9.5 ± 0.6	3.2	0.5	0.32	76
Steam explosion and enzymes; CE/HC; wheat straw (e)	8.4 ± 0.6	2.8	0.5	0.28	67
Organosolv process and enzymes, CE, beech (f)	11.5 ± 0.4	4.2		0.42	100
Organosolv process and enzymes, HC, beech (g)	0.1 ± 0.0	—	—	—	—
P. putida KT2440_*xylAB*					
Glucose (reference)	12.4 ± 0.3	4.1	0.7	0.41	98
Sulfuric acid; CE/HC; beech (a)	0.2 ± 0.1	—	—	—	—
Sulfuric acid; CE/HC; spruce (b)	0.2 ± 0.0	—	—	—	—
Hydrochloric acid; HC; rice hulls (c)	11.5 ± 0.7	3.8	0.4	0.41	90
Steam explosion and enzymes; CE/HC, miscanthus (d)	11.3 ± 0.5	3.8	0.4	0.38	90
Steam explosion and enzymes; CE/HC; wheat straw (e)	11.7 ± 0.8	3.9	0.5	0.39	93
Organosolv process and enzymes, CE, beech (f)	12.6 ± 0.3	4.2		0.42	100
Organosolv process and enzymes, HC, beech (g)	0.1 ± 0.0	—	—	—	—

Abbreviation: RCHC, relative carbon hydrolyzate conversion.

[a]Bacterial biomass (X) to substrate (S) ratio, substrate: 10 g/L carbohydrate.

[b]Ratio of biomass of respective cultivation to the cultivation with *P. putida* KT2440 (wild type) and glucose as carbon source.

Maximum achieved biomass of *P. putida* KT2440 wild type decreased slightly already upon addition of formic acid starting at a concentration of 1 g/L from a maximum OD_{600} 12.3 to 11.5 (Figure 2a). At the highest applied formic acid concentration of 10 g/L the cells reached a maximum OD_{600} of 8.4. The recombinant strain was more sensitive to formic acid than the wild type. Cell growth decreased upon addition of formic acid starting at a concentration of 1 g/L (maximum OD_{600} = 10.5). At 5 g/L formic acid the maximum OD_{600} was 4.7 and at 10 g/L the maximum OD_{600} was 2.9. Acetic acid had no negative influence on the growth of the two *P. putida* KT2440 strains (Figure 2b) until the highest applied acid concentration of 10 g/L. Furthermore, the strains metabolized acetic acid and partially used it for growth (Arnold, Tews, Tews, Kiefer, Henkel, & Hausmann, 2019). In detail, compared to cultivation without acetic acid (maximum OD_{600} = 12.4), the wild type reached a maximum OD_{600} of 14.6 and 15.1 at acetic acid concentrations of 2.5 and 10 g/L, respectively. *P. putida* KT2440_*xylAB* showed similar growth characteristics as the wild type strain and reached a maximum OD_{600} of 12.3, 15.2, and 15.9 at acetic acid concentrations of 0, 2.5, and 10 g/L, respectively. The addition of acetic acid had no effect on the length of the lag phase.

Furfural causes an extension of the lag phase in low concentrations and a reduction in cell growth in high concentrations (Figure 2c). There was hardly any difference between wild type and recombinant strain. The lag phase was extended starting from a threshold of 0.4 g/L furfural concentration (lag phase = 6 hr) and at a concentration of 4 g/L, cell growth started with a delay of 24 hr after inoculation. No growth was detectable over a period of 5 days at a concentration of 5 g/L furfural (data not shown). Similar to furfural, the addition of HMF resulted in an extension of the lag phase and, at a higher concentration, in a decrease in cell growth (Figure 2d). In detail, the lag phase of *P. putida* KT2440 wild type was prolonged starting from a threshold value of 1.2 g/L HMF concentration (lag phase = 4 hr) and the lag phase lasted for 8 hr at an HMF concentration of 2.4 g/L and approx. 52 hr at an HMF concentration of 4.8 g/L. A decrease in the maximum OD_{600} could be observed only at higher HMF concentrations starting at 2.8 g/L with a maximum OD_{600} of 7.7. No growth could be detected at the highest applied HMF concentration of 5.2 g/L. *P. putida* KT2440_*xylAB* proved to be slightly more resistant to HMF. When HMF was added, the lag phases were also extended, but to a lesser extent than for the wild type. In addition, the maximum OD_{600} almost did not decrease up to HMF concentrations of 4.8 g/L and remained constant at approx. 12.0. As with the wild type,

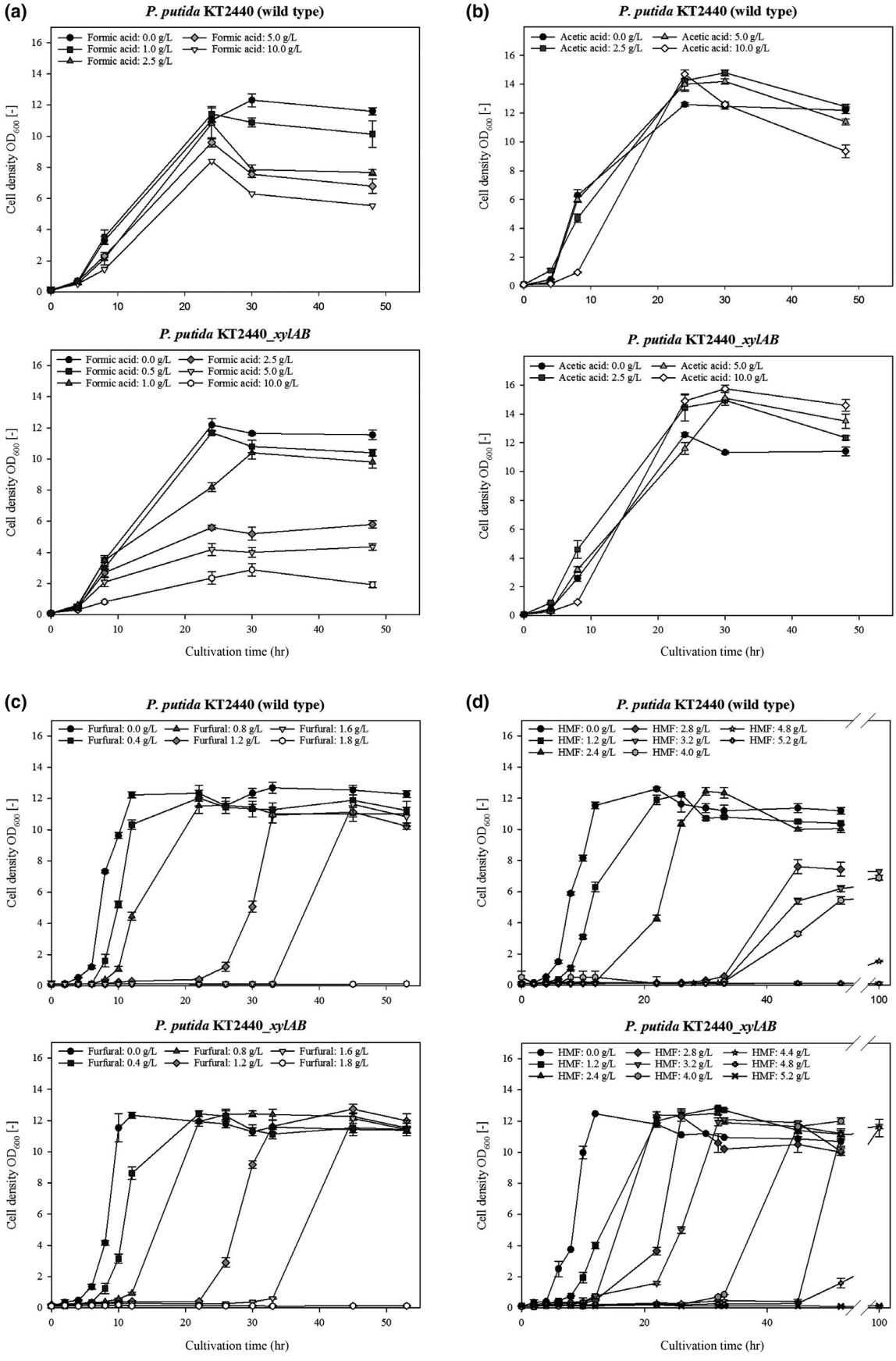

FIGURE 2 Time courses of OD_{600} during cultivation with *Pseudomonas putida* KT2440_*xylAB* and wild type by using glucose as a primary carbon source and adding formic acid (a), acetic acid (b), furfural (c), and hydroxymethylfurfural (HMF; d) to the medium

no growth was detected at HMF concentration of 5.2 g/L over a period of 5 days.

3.3 | Fed-batch bioreactor cultivations with lignocellulose hydrolyzates as carbon source

Pseudomonas putida KT2440_*xylAB* was able to grow with several hydrolyzates and reached comparable growth rates as with glucose. Building on this, a fed-batch process was performed in a 2 L bioreactor with wheat straw hydrolyzates as carbon sources. Furthermore, the results of the shaking flask cultivations led to the hypothesis that the recombinant strain only metabolizes xylose once glucose has been consumed. To confirm this hypothesis, fed-batch experiments were performed.

Observed growth shows a preferential metabolization of glucose, followed by xylose and lastly arabinose (Figure

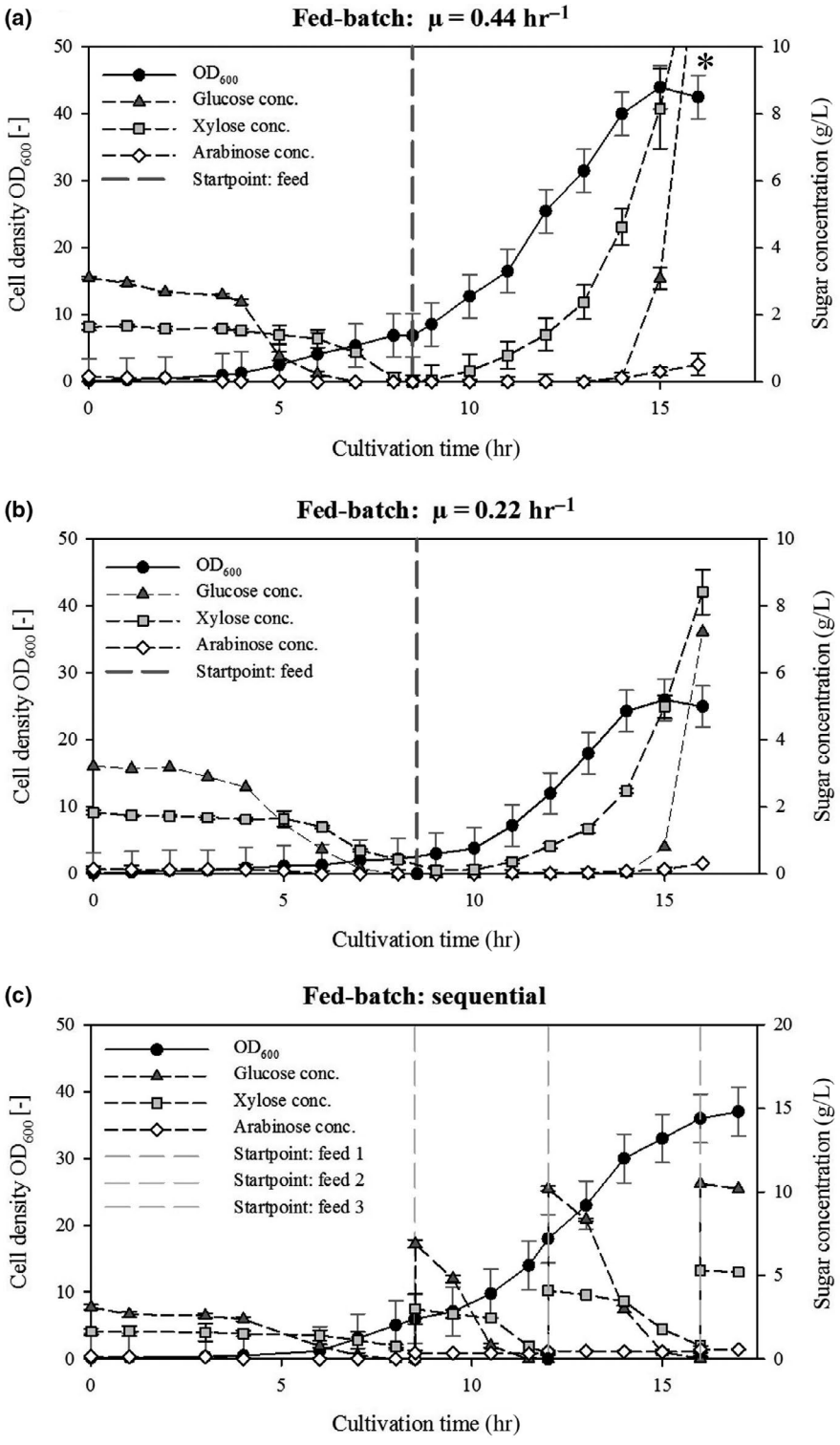

FIGURE 3 Time courses of OD_{600} and sugar concentration during fed-batch bioreactor cultivations with *Pseudomonas putida* KT2440_*xylAB*. (a) Calculated growth rate of $\mu = 0.44$/hr, (b) $\mu = 0.22$/hr, (c) sequential feeding. *Concentration after 16 hr: glucose 23.9 g/L, xylose 13.2 g/L

3a), in accordance with previous observations (Figure 1). The feed was started when glucose and xylose were almost consumed. As expected, the strains almost did not metabolize xylose and arabinose in the presence of glucose. Xylose was accumulated in the medium and after 15 hr a concentration of 8.2 g/L could be detected.

To solve the issue of sugar accumulation, a lower growth rate of $\mu = 0.22$/hr was applied for the next experiment (Figure 3b) to potentially enable uptake of xylose and arabinose. However, similar results regarding sugar accumulation were obtained. As a consequence, feed medium was sequentially added in the next fed-batch experiment. This feed medium was only added when xylose was almost depleted (Figure 3c). The applied feed profile did indeed lead to complete metabolization of relevant sugars xylose and arabinose. Furthermore, during metabolization of xylose, growth could be detected and the strain grew without any relevant lag phase. A maximum OD_{600} of 35 was achieved after 15 hr.

4 | DISCUSSION

4.1 | Lignocellulose hydrolyzates as carbon source for *P. putida* KT240_*xylAB*

Pseudomonas putida KT2440_*xylAB* reached similar growth rates with miscanthus and wheat straw hydrolyzates treated with steam explosion and enzymes (hydrolyzate d + e) as compared with glucose. However, it should be noted that for the combination of steam explosion and enzymatic hydrolysis, depolymerization of CE and HC to sugars is incomplete and may be optimized in the future. For the applied wheat straw, conversion of CE (38%) and HC (41%) to the respective monosaccharides is reported (Schläfle et al., 2017). However, if more severe process conditions for steam explosion are applied to obtain a better enzymatically convertible substrate, this may result in increased levels of HMF and furfural, which may have a negative effect on the applicability of the hydrolyzate. In this case, a view into polymer-degrading microorganisms could be worthwhile, including suitable yeasts, fungi, or specialized bacteria such as *Cellvibrio japonicus* (Gardner, 2016; Gardner & Keating, 2010; Horlamus et al., 2019). Furthermore, a genome-edited derivative of *P. putida* KT2440 was recently constructed, which was able to use cellobiose as the carbon source (Dvořák & de Lorenzo, 2018).

The results with hydrochloric acid-depolymerized samples (hydrolyzate c) are especially interesting, since only HC-derived sugars were used. As such, conversion of contained sugars to biomass is significantly lower for the wild type, as no conversion of xylose and arabinose is observed. In a future study, performance of these hydrolyzates could also be investigated for bioreactor cultivations.

When applying the hydrolyzates obtained after digestion with sulfuric acid (hydrolyzate a + b) as the carbon source, no growth of *P. putida* KT2440_*xylAB* and wild type was detected. This hydrolysis process was initially designed for a nonbiochemical value chain. The goal was to obtain high sugar concentrations in the hydrolyzate after short reaction times and to convert the sugars in further reaction steps to furfural and HMF. Consequently, the chosen hydrolysis reaction conditions were chosen were more severe compared to hydrolysis processes designed for fermentable sugar production. Therefore, more fermentation inhibitors are present in the hydrolyzate of this sulfuric acid hydrolysis process. High concentrations of furfural and HMF were identified as main critical points for *P. putida* KT2440. Besides individual inhibitory effects, the combined action of different inhibitors is still unknown for *P. putida* KT2440 and could be a further target of research. This is apparent for hydrolyzate b, where the HMF and furfural are each not present at critically inhibiting concentrations, yet, no growth could be observed.

For detoxification of hydrolyzates, many different physical, chemical, and biochemical processes have been developed and summarized (Chandel et al., 2011; Jönsson et al., 2013). The high concentrations of HMF and furfural do not have to be a disadvantage, as both are important basic building block chemicals (Steinbach, Kruse, & Sauer, 2017). For this reason, a worthwhile process strategy could be to combine chemical and biotechnological methods. Furfural and HMF should be separated from hydrolyzates firstly to obtain them as marketable products and secondly to get detoxified hydrolyzates which can be applied as the carbon source for microorganisms.

The CE fraction of the organosolv process (hydrolyzate f) was a highly suitable carbon source and similar growth rates as with glucose were achieved. No growth could be measured with the HC fraction (hydrolyzate g), although the measured organic acid or furfural aldehyde concentrations were not critically high. A potential reason for that could be lignin-derived phenolic compounds, which are not determined in this study but should be a topic for future research, because phenols are also well-known fermentation inhibitors (Palmqvist & Hahn-Hägerdal, 2000).

In a previous study, beech hydrolyzates from the same organosolv process as applied in this study were used as carbon source for organic acid production with filamentous fungi (Dörsam et al., 2017). In contrast to *P. putida* KT2440, *Aspergillus oryzae* was able to grow with HC hydrolyzates as the carbon source. A final malic acid concentration of 5.8 g/L and an overall production rate of 0.03 g/(L*hr) in a bioreactor cultivation with 99.5 g/L HC sugars was reported. *Rhizopus delemar* was more sensitive to inhibitors than *A. oryzae* and a production of organic acids with the HC fraction as the carbon source was not possible.

4.2 | Inhibitors

Pseudomonas putida KT2440_*xylAB* and wild type proved to tolerate acetic acid up to the highest measured concentration

of 10 g/L after neutralization of the medium. This is a major advantage for applying this strain as host for lignocellulose biorefinery since most hydrolyzates contain acetic acid below a concentration of 10 g/L originating from HC (Chandel et al., 2011; Palmqvist & Hahn-Hägerdal, 2000). Both strains were however less tolerant to formic acid than to acetic acid and growth was weakened starting from a concentration of 1.0 g/L.

As most hydrolyzates contain only a small amount of formic acid clearly below 1.0 g/L (Chandel et al., 2011; Palmqvist & Hahn-Hägerdal, 2000), this is an issue only in high-efficiency fed-batch bioprocesses, where an accumulation of formic acid after prolonged feeding would occur. Furthermore, the recombinant strain was more sensitive to formic acid than the wild type. It should be noted that the additional plasmid and the expression of additional genes represents a metabolic burden for the bacteria. This may not have a serious effect under moderate cultivation conditions but may lead to reduced growth in the presence of another stress factor. Furfural and HMF are identified as critical components, since a negative influence on growth could be identified starting from a furfural concentration of approx. 0.4 g/L and an HMF concentration of 1.2 g/L. This was not reflected in a deterioration in growth rates, but in an extension of the lag phase. This is consistent with results obtained in a study in which a lag phase of 24 hr was observed during the cultivation of *P. putida* KT2440 with lignocellulose hydrolyzates supplemented with 2 g/L HMF and 1 g/L furfural. An explanation for this is the metabolization of furfural aldehydes to less toxic dead-end alcohol counterparts (furfuryl alcohol and HMF furfuryl alcohol) practiced by many microorganisms, which has been reported in the past (Guarnieri, Ann Franden, Johnson, & Beckham, 2017). Furthermore, in theory, part of the highly reactive furfural and HMF could have formed less toxic macromolecules over time of the cultivation. However, under the applied experimental conditions, it was verified that this was not the case (data not shown).

In case of microbial biorefinery, organic acids and furan aldehydes are mainly discussed as inhibitors, although they consist of carbon to a considerable extent. As a consequence, a future lignocellulose strain should not only have a high resistance to these compounds but also be able to utilize them as carbon source. *P. putida* KT2440_*xylAB* and wild type were able to metabolize acetic acid. With an amount of 2.5 g/L, the maximum OD_{600} increased from 12.4 to 14.6. Since *P. putida* KT2440 wild type is not able to use furfural and HMF as substrates for growth, Guarnieri et al. (2017) engineered a strain via genomic integration of the *hmf* gene cluster. Consequently, the strain metabolized HMF and furfural via the intermediate 2-furoic acid. This constructed strain grew on HMF and furfural up to a concentration of 1 g/L. In summary, the results show that the HMF and furfural content in hydrolyzates is a key criterion for applying lignocellulosic hydrolyzates as a carbon source for *P. putida* KT2440.

4.3 | Fed-batch cultivation in a bioreactor

During cultivation with hydrolyzate, a diauxic-like grown pattern with a nonsimultaneous consumption of different sugars was observed. With sufficient carbon supply, the recombinant strain metabolized glucose but almost no decrease in xylose and arabinose could be detected. Considering an envisioned high-efficiency bioprocess, this is an issue that needs to be addressed in terms of complete consumption of sugars and carbon efficiency as well as potentially inhibitory effects of accumulation sugars.

Reduction of added sugars by a decrease in applied growth rate of exponential feeding did not lead to a significant consumption of xylose and arabinose confirming the presence of a diauxic-like growth behavior. As a potential strategy to overcome this issue, a stepwise fed-batch process was investigated. It was shown that not only glucose but also xylose as a carbon source was consumed by the microorganisms. Another possibility to overcome this issue could be to employ a bacterial consortium with glucose-negative strains for metabolizing HC sugars or engineered strains for simultaneous consumption of sugars (Dvořák & de Lorenzo, 2018). Le Meur, Zinn, Egli, Thöny-Meyer, & Ren (2012) transformed *P. putida* KT2440 with *xylAB* genes and then carried out bioreactor cultivation with xylose and octanoic acid. However, only limited biomass concentrations of 2.7 g/L were reported while the production of medium-chain length polyhydroxyalkonoates was the focus of this study.

For application of *P. putida* within the frame of a bio-based economy, a fed-batch process for simultaneous consumption of all contained sugars is envisioned. In the future, along with engineered processes or strains, a model could be developed to evaluate the process economically and ecologically. Altogether, this study represents a first step and proof-of-concept toward establishing *P. putida* KT2440 as a platform for bioconversion of lignocellulose hydrolyzates.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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