BIOTECHNOLOGICAL CONVERSION OF LIGNOCELLULOSE HYDROLYZATES

- MODEL MICROORGANISMS FOR A BIO-BASED ECONOMY -

Dissertation to obtain the doctoral degree
of Natural Sciences (Dr. rer. nat.)

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from Erlangen
2020

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Submitted on: 02.03.2020

Oral examination on: 23.07.2020

Great things are not done by impulse, but by a series of small things brought together.

Vincent van Gogh

Acknowledgements

I would like to express my deep appreciation to the following people for their guidance and support through my research project:

Prof. Dr.-Ing Rudolf Hausmann for providing me this precious opportunity to be part of his research group. I would like to thank him for his excellent scientific supervision, his continuous guidance and support.

Dr.-Ing. Marius Henkel for his profound scientific knowledge and his advice in the right moments which helped me to move forward and to progress in my work.

My dear colleagues for the friendly and family-like atmosphere which guaranteed a great working atmosphere: Dipl.-Biol. Anja Sander, M. Eng. Fabiola Weggemann, Dr. rer. nat. Kambiz Morabbi Heravi, M. Sc. Mareen Geissler, M. Sc. Philipp Noll, Dipl.-Biol. Ramona Faas (neé Bosch) and M. Sc. Stefanie Arnold.

Dr. Stephen P. Buchner and **Dr. Lovemore Kunorozva** for spending time and energy on proofreading my dissertation and my manuscripts.

All the hard-working and extremely motivated students who are involved in this research project by completing their bachelor thesis, master thesis or project: B. Sc. Antonia Huppert, M. Sc. Ines Kühle, M. Sc. Inga Müller (neé Spindelböck), B. Sc. Jan Michler, M. Sc. Lucie Scheske, M. Sc. Lukas Gerstweiler, B. Sc. Nico Piskors, B. Sc. Pauel Grießhaber, B. Sc. Philipp Kohl, B. Sc. Sandra Lichtenberger.

Project partners and academic partners, many thanks for the trusting and productive cooperation Dr. rer. nat. Andreas Wittgens, Dr. rer. nat. Claudia Oellig, M. Sc. David Steinbach, Dr. rer. nat. Frank Rosenau, Dr. rer. nat. Julia Hofmann, Dr. rer. nat. Sandra Schläfle and M. Sc. Yan Wang.

Dr. rer. nat. Frank Rosenau for their participation as members of my Thesis Advisory Committee and their recommendations and support. **Prof. Dr. rer. nat. Christoph Syldatk** for his willingness to be my second reviewer.

Prof. Dr. rer. nat. Gertrud Morlock, **Prof. Dr. hab. Grażyna Lewandowicz**, **PD Dr. rer. nat. Ralf Paetzold**, **Prof. Dr. hab. Tomasz Jankowski**, **Jun.-Prof. Dr. rer. nat. Yanyan Zhang** for inspiring and encouraging me during my studies.

Prof. Carlos Ariel Cardona for giving me the opportunity to perform my foreign research exchange in his department, **Dr. rer. nat. Annette Weidtmann** for the coordination the stay abroad and **M. Sc. Sebastián Serna Loaiza** for his support during my stay in Colombia.

The Ministry of Science, Research and the Arts of Baden-Württemberg and the Fachagentur Nachwachsende Rohstoffe e.V. for funding this project. Moreover, I would like to acknowledge the **graduate program BBW ForWerts** for organizing all the excellent and interesting research activities.

All my **fantastic friends**, my **great hockey team**, my **cycling group** and "**Casa masa loca**", for always believing in me and for creating so much fun.

To my phenomenal partner **Maliheh Vahidinasab** for her continuous love and care and for being a great lab partner and researcher. Many thanks to **Vahidinasab family (Fahimeh, Effat and Mohammad)** for their great support.

My family, Elisabeth, Gertraud, **Julia**, **Norbert** and **Simon,** thank you so much for your continuous accompanying and encouragement.

Publications and congress contributions

Peer reviewed original publications

Wang Y*, Horlamus F*, Henkel M, Kovacic F, Schläfle S, Hausmann R, Wittgens A, Rosenau F
GROWTH OF ENGINEERED *PSEUDOMONAS PUTIDA* KT2440 ON GLUCOSE, XYLOSE AND ARABINOSE: HEMICELLULOSE HYDROLYSATES AND THEIR MAJOR SUGARS AS SUSTAINABLE CARBON SOURCES
Global Change Biology Bioenergy 2019, 11:249–259. doi: 10.1111/gcbb.12590

Horlamus F*, Wang Y*, Steinbach D, Vahidinasab M, Wittgens A, Rosenau F, Henkel M, Hausmann R
POTENTIAL OF BIOTECHNOLOGICAL CONVERSION OF LIGNOCELLULOSE HYDROLYZATES BY *PSEUDOMONAS PUTIDA* KT2440 AS MODEL ORGANISM FOR A BIO-BASED ECONOMY
Global Change Biology Bioenergy 2019, 11:1421–1434. doi: 10.1111/gcbb.12647

Horlamus F, Wittgens A, Noll P, Michler J, Müller I, Weggenmann F, Oellig C, Rosenau F, Henkel M, Hausmann R
ONE-STEP BIOCONVERSION OF HEMICELLULOSE POLYMERS TO RHAMNOLIPIDS WITH *CELLVIBRIO JAPONICUS*: A PROOF-OF-CONCEPT FOR A POTENTIAL HOST STRAIN IN FUTURE BIOECONOMY
Global Change Biology Bioenergy 2019, 11:260–268. doi: 10.1111/gcbb.12542

*Co-first authorship

Erfindungsmeldung

Henkel M, Horlamus F, Spindelböck I, Weggenmann F, Wittgens A, Rosenau F, Hausmann R
BIOTECHNOLOGISCHE HERSTELLUNG VON RHAMNOLIPIDEN (UND ANDEREN PRODUKTEN) AUF BASIS VON LIGNOCELLULOSEPOLYMEREN (CELLULOSE, XYLANE) MIT DEM BAKTERIUM *CELLVIBRIO JAPONICUS*, 29.11.2016

Selected conference talks

Horlamus F, Wang Y, Wittges A, Rosenau F, Henkel M, Hausmann R
DEVELOPMENT OF RECOMBINANT BACTERIA STRAINS FOR THE PRODUCTION OF BIOSURFACTANTS FROM LIGNOCELLULOSIC HYDROLYZATES
29th Colombian Congress of Chemical Engineering and Related Careers "Engineering as Engine for the Reindustrialization of Colombia", 19.10.2017, Manizales, Colombia

Horlamus F, Geißler M, Henkel M, Hausmann R
SUGAR ANALYTICS WITH HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY (HPTLC)
1st BBW ForWerts Summer School "Bioeconomy: The Guarantor for Sustainable Development?", 27.07.2015, Höchst, Germany

Selected poster presentations

Horlamus F, Weggenmann F, Hofmann J, Fredrich B, Beuker J, Wittgens A, Rosenau F, Schomburg D, Henkel M, Hausmann R
HETEROLOGOUS BIOSYNTHESIS OF RHAMNOLIPIDS - PROCESS OPTIMIZATION AND NOVEL METABOLIC ROUTES
BioProcessingDays 2018 "Produktion (Contract Manufacturing) – Zellkultur – Single Use", 19-21.02.2018, Recklinghausen, Germany

Horlamus F, Wang Y, Wittges A, Rosenau F, Griesshaber P, Henkel M, Hausmann R
DEVELOPMENT OF HEMICELLULOSE METABOLIZING BACTERIA STRAINS FOR PRODUCTION OF RECOMBINANT PROTEINS
2nd International Bioeconomy Congress, 12-13.09.2017, Stuttgart, Germany

Horlamus F, Wang Y, Rosenau F, Henkel M, Hausmann R
DEVELOPMENT OF A SELF-REGULATING MICROBIAL SYSTEM FOR THE PRODUCTION OF RECOMBINANT PROTEINS FROM RENEWABLE RAW MATERIALS
DECHEMA Himmelfahrtstagung "Models for Developing and Optimising Biotech Production", 22-24.05.2017, Neu-Ulm, Germany

Horlamus F, Wang Y, Rosenau F, Henkel M, Hausmann R
SELF-REGULATING BACTERIAL CONSORTIA: A NOVEL ROUTE FOR ACCESSING LIGNOCELLULOSE - THE WORLD´S MOST ABUNDANT RENEWABLE RESOURCE
2nd Status Seminar and Interim Evaluation of the Bioeconomy Research Program Baden-Württemberg,16.10.2016, Stuttgart, Germany

Abstract

Lignocellulose has substantial potential as a carbon source in a bio-based economy. It is the most abundant renewable raw material on earth and is available in large quantities as waste from the agriculture, food and wood industry. It is composed mainly of the polymers lignin, cellulose and hemicellulose. In contrast to glucose derived from cellulose, hemicellulose sugars often remain unused although 60 billion tons of hemicelluloses are produced annually. Hemicelluloses are a group of heterogeneous polysaccharides consisting of different monomers such as D-xylose, D-arabinose, D-mannose and D-galactose. Lignocellulose is mostly depolymerized in order to obtain fermentable sugars. During the depolymerization process, inhibitors such as organic acids or furan aldehydes can be formed or released, which could be problematical for biotechnological processes.

The aim of this thesis was to develop and evaluate bacterial-based biotechnological processes capable of using hemicellulose sugars as a source of carbon. First, *Pseudomonas putida* KT2440 was chosen. Pseudomonades are claimed as a promising chassis in biotechnology due to their versatile and robust metabolism. Unlike other Pseudomonades, the strain KT2440 is classified as biosafety level 1 in the American Type Culture Collection (ATCC). However, these bacteria can metabolize glucose as the only lignocellulose monosaccharide. *Cellvibrio japonicus* was the second selected bacterium. This strain is not yet established as a microbial host in biotechnology, but can degrade a huge portfolio of plant cell wall polysaccharides and is also classified as biosafety level 1 in ATCC.

The topic of the first publication was to engineer *P. putida* KT2440 strains for metabolizing the hemicellulose monosaccharides xylose and arabinose and characterize their growth behavior. Initially, an arabinose metabolizing strain with the *araBAD* operon and a xylose

metabolizing strain with *xylAB* operon was constructed. Later on, these strains were cultivated in minimal salt medium with glucose, xylose and arabinose as carbon sources in Erlenmeyer flasks. The recombinant *P. putida* KT2440 strains metabolized xylose and arabinose with high growth rates comparable to glucose. It turned out that both engineered strains were able to grow on both pentoses as well as on mixtures of glucose xylose and arabinose.

The intent of the second publication was to evaluate *P. putida* KT2440 as a platform model organism for bioconversion of lignocellulose hydrolyzates. Strains were cultivated in minimal salt medium with several hydrolyzates as carbon source in Erlenmeyer flask and bioreactor. In addition, the growth-inhibiting effect of major toxic substances contained in lignocellulose hydrolyzates on *P. putida* KT2440 was analyzed via cultivation experiments. Several suitable hydrolyzates were figured out for this strain. Formic acid and acetic acid proved to be relatively unproblematic under pH-neutral conditions, whereas furfural and hydroxymethylfurfural (HMF) had a negative effect on the bacterial growth. A diauxic-like growth behavior was revealed via fed-batch bioreactor cultivations, since pentoses were almost not consumed with sufficient glucose supply. Consequently, feed-medium was added step-by-step in the next experiment. The applied feed profile did lead to an almost complete metabolization of xylose.

The purpose of the third publication was to evaluate *C. japonicus* as a potential host strain for the one-step bioconversion of xylans into rhamnolipids. Cultivation experiments were performed in Erlenmeyer flasks filled with minimal salt medium and containing different carbon sources. Furthermore, the strain was transformed with the plasmid pSynPro8oT carrying *rhlA* (encodes acetyltransferase) and *rhlB* (encodes rhamnosyltransferase I) to complete the rhamnolipid metabolism. The strain grew on all main lignocellulose monosaccharides as well as, on different xylans. Mono-rhamnolipids were produced with the engineered strain using xylans as carbon source. This is particularly interesting as most industrially relevant bacteria are not able to depolymerize wood polymers. As the product yields were quite low, there are still many challenges in order to achieve an economically efficient process. Nevertheless, to the best of our knowledge, it is the first published one-step bioconversion of hemicellulose polymers into rhamnolipids.

In total, *P. putida* KT2440 turned out as a flexible and powerful model organism and two xylose and arabinose metabolizing strains were constructed. Moreover, bioreactor cultivations with lignocellulose hydrolyzates were performed and a feeding strategy to overcome diauxic-like growth behavior was presented. A proof of concept for a one-step bioconversion of xylans into rhamnolipids with a recombinant *C. japonicus* strain was successfully demonstrated.

Zusammenfassung

Im Rahmen einer biobasierten Wirtschaft wird Lignocellulose als Kohlenstoffquelle ein großes Potential zugesprochen. Sie ist der am häufigsten vorkommende nachwachsende Rohstoff auf unserer Erde und steht in großen Mengen als Abfallstoff aus der Agrar-, Lebensmittel- und Holzindustrie zur Verfügung. Lignocellulose besteht hauptsächlich aus den Polymeren Lignin, Cellulose und Hemicellulose. Im Gegensatz zu der aus der Cellulose stammenden Glucose werden die Hemicellulose-Zucker selten als Kohlenstoffquelle in biotechnologischen Prozessen verwendet, obwohl jährlich ca. 60 Milliarden Tonnen Hemicellulosen anfallen. Hemicellulosen sind eine Gruppe von heterogenen Polysacchariden, welche hauptsächlich aus den Monosacchariden D-Xylose, L-Arabinose, D-Mannose D-Glucose und D-Galactose zusammengesetzt sind. Um fermentierbare Zucker aus der Lignocellulose zu erhalten, muss diese meist depolymerisiert werden. Bei diesem Verzuckerungsprozess können für biotechnologische Prozesse problematische Stoffe wie z.B. organische Säuren oder Furanaldehyde gebildet oder freisetzet werden.

Ziel dieser Arbeit war es, bakteriell basierte biotechnologische Prozesse zu entwickeln und zu bewerten, die Hemicellulose-Zucker als Kohlenstoffquelle nutzen können. Zum einen wurde der Bakterienstamm *Pseudomonas putida* KT2440 ausgewählt. Pseudomonaden gelten aufgrund ihres vielseitigen und robusten Stoffwechsels als vielversprechende Biokatalysatoren. Im Gegensatz zu vielen anderen Pseudomonaden ist der Stamm KT2440 in der ATCC (American Type Culture Collection) unter der Risikogruppe 1 eingeordnet. Allerdings können Pseudomonaden außer Glucose keine weiteren Lignocellulose-Zucker als Kohlenstoffquelle nutzen. Als zweiter Bakterienstamm wurde *Cellvibrio japonicus* ausgewählt. Dieser Stamm ist als Wirt für biotechnologische Prozesse nicht etabliert, ist aber

in der Lage**,** ein großes Portfolio an Pflanzenzellwand-Polysacchariden abzubauen und ist der Risikogruppe 1 zugeordnet.

Das Thema der ersten Publikation war die Konstruktion von Xylose- und Arabinose-verstoffwechselnden *P. putida* KT2440-Stämmen und die anschließende Charakterisierung ihres Wachstumsverhaltens. Zunächst wurde jeweils ein Stamm zur Metabolisierung von Xylose und Arabinose konstruiert. Hierzu wurde das *xylAB*-Operon und das *araBAD*-Operon von *Escherichia coli* verwendet. Zur Analyse des Wachstumsverhaltens wurden die Stämme daraufhin in Minimalmedium und mit Glucose, Xylose und Arabinose als Kohlenstoffquelle in Erlenmeyerkolben kultiviert. Die rekombinanten *P. putida* KT2440 Stämme wuchsen mit Xylose und Arabinose mit ähnlich hohen Wachstumsraten wie mit Glucose. Zudem konnten beide Stämme die jeweils andere Pentose verstoffwechseln und es konnten Kultivierungen mit Mischungen aus Glucose, Xylose und Arabinose erfolgreich durchgeführt werden.

Ziel der zweiten Publikation war es, das Potenzial von *P. putida* KT2440 als Biokatalysator für die Biokonversation von Lignocellulose-Hydrolysaten zu bewerten. Die Stämme wurden in Minimalmedium mit verschiedenen Hydrolysaten als Kohlenstoffquelle in Erlenmeyerkolben und im Bioreaktor kultiviert. Darüber hinaus wurde mittels Kultivierungsversuchen die wachstumshemmende Wirkung von in Lignocellulose-Hydrolysaten enthaltenen toxischen Substanzen auf *P. putida* KT2440 untersucht. Es konnten mehrere geeignete Hydrolysate für diesen Stamm ausgemacht werden. Ameisensäure und Essigsäure erwiesen sich als relativ unproblematisch unter pH-neutralen Bedingungen, wohingegen Furfural und Hydroxymethylfurfural (HMF) schon bei niedrigen Konzentrationen einen negativen Effekt auf das Wachstum von *P. putida* KT2440 hatten. Mittels eines Fed-Batch-Prozesses im Bioreaktor mit Lignocellulose-Hydrolysaten wurde ein diauxi-ähnliches Wachstumsverhalten nachgewiesen. Bei ausreichend verfügbarer Glucose wurde die Xylose kaum verstoffwechselt. Als mögliche Strategie zur Lösung dieses Problems wurde der Feed stufenweise dazugegeben. Dies führte zu einer nahezu vollständigen Verstoffwechselung von Xylose.

Im Rahmen der dritten Veröffentlichung wurde ein Prozess zur einstufigen Biokonversion von Hemicellulose-Polymeren zu Rhamnolipiden mit dem Bakterium *C. japonicus* als Wirtstamm getestet. *C. japonicus* wurde in Minimalmedium zusammen mit verschiedenen

Kohlenstoffquellen in Erlenmeyerkolben kultiviert. Um den Rhamnolipidstoffwechsel zu vervollständigen, wurde der Stamm mit dem Plasmid pSynPro8oT mit *rhlA* (kodiert Acetyltransferase) und *rhlB* (kodiert Rhamnosyltransferase I) transformiert. *C. japonicus* wuchs auf allen wichtigen Lignocellulose-Monosacchariden sowie auf verschiedenen Xylanen. Mit dem rekombinanten Stamm und Xylan als Kohlenstoffquelle konnte Mono-Rhamnolipid hergestellt werden. Dies ist insbesondere interessant, da die meisten industriell relevanten Bakterien nicht in der Lage sind, Holzpolymere zu depolymerisieren und zu verstoffwechseln. Jedoch erwies sich die Transformation als schwierig und es konnten nur niedrige Produktausbeuten erzielt werden. Aufgrund dessen sind noch viele Herausforderungen zu bewältigen, um einen wirtschaftlich rentablen Prozess zu erhalten. Nichtsdestotrotz handelt es sich nach unserem Wissen um die erste einstufige Biokonversation von Hemicellulose-Polymeren zu Rhamnolipiden.

Zusammenfassend, erwies sich *P. putida* KT2440 als flexibler und leistungsfähiger Wirtsorganismus. Xylose und Arabinose metaboliserende Stämme wurden konstruiert und Prozessstrategien für Bioreaktor-Kultivierungen wurden entwickelt. Des Weiteren konnte ein Proof-of-Concept für eine einstufige Biokonversion von Xylanen zu Rhamnolipiden mit einem transformierten *C. japonicus* Stamm erfolgreich erbracht werden.

Table of contents

Abbreviations and symbols

approx.	approximately
ATP	adenosine triphosphate
cAMP	Cyclic AMP
CEs	carbohydrate esterases
C. japonicus	*Cellvibrio japonicus*
CBP	Consolidated bioprocessing
CRP	cAMP receptor protein
DNA	deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms)
EC	enzyme commission (number)
E. coli	*Escherichia coli*
ED	Entner-Doudoroff pathway
e.g.	lat. exempli gratia (for example)
Gcd	glucose dehydrogenase
GHs	glycoside hydrolases
HMF	hydroxymethylfurfural
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin-layer chromatography
IPTG	isopropyl β-D-1-thioglactopyranoside
KDPG	2-keto-3-deoxy-6-phosphogluconate
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	lysogeny broth
MS	mass spectrometry
NADH / NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NCIMB	National Collection of Industrial Food and Marine Bacteria
n.d.	not determined
OD	optical density
P_i	inorganic phosphate
PLs	polysaccharide lyases

pO_2	dissolved oxygen (%)
RCHC	relative carbon hydrolyzate conversion
PPP	pentose phosphate pathway
P. putida	*Pseudomonas putida*
PTS	phosphoenolpyruvate: carbohydrate phosphotransferase system
Rpm	revolutions per minute
SSF	Simultaneous saccharification and fermentation and consolidated bioprocessing
UDP	uridine diphosphate
UV	ultraviolet (light / radiation)
XRDH	xylose reductase-xylitol dehydrogenase pathway
Yx|s	biomass to substrate yield

μ	specific growth rate
g	gravitational acceleration
hr	(process- or reaction-) time
T	temperature
v/v	volume per volume (concentration)

1 Introduction

In recent years much attention has been given to the study of renewable materials as substitutes for fossil resources. Major driving forces for this research field are the reduction of the global carbon dioxide emissions, the diminution of dependence on crude oil and the development of rural areas and the agricultural sector (Jørgensen et al. 2007; Lange 2007; Loeffler et al. 2018). Currently, approximately three quarters of fossil fuels are used for power and heating applications, one quarter for transportation, and a few percent for production of chemicals (Lange 2007). A variety of resources such as biomass, wind and solar power are available for power and heat supply. For producing chemicals and materials, however, there are hardly any alternatives to fossil resources other than renewable materials (FitzPatrick et al. 2010).

The first generation of biofuels was produced from easily accessible parts of the plants, such as starch and vegetable oils. Mostly plants such as sugar cane, cereals and maize were used. Bearing in mind the world population growth, this is not a sustainable solution because it competes with food production (Lange 2007; Naik et al. 2010). An alternative is lignocellulose, being the most abundantly available renewable raw material on the planet. Lignocellulose is the structural framework of woody plant cell walls and consists mainly of the polymers cellulose, hemicellulose(s) and lignin(s). There are many different sources for the raw materials, such as wood residues, grasses, waste paper, agricultural residues, food-industry residues and municipal solid wastes (Anwar et al. 2014; Jørgensen et al. 2007; Naik et al. 2010; Saini et al. 2015). Therefore, there has been increased interest in lignocellulose in recent years. According to the database of the European Patent Office, Espacenet, and the Scopus database, the total number of 6097 patents and 11212 publications containing

lignocellulose as a term the in title/abstract has increased fourfold and tenfold respectively in the last 20 years.

In natural processes, lignocellulosic materials such as fallen tree trunks are completely metabolized by a consortium of decomposing fungi and bacteria (Cragg et al. 2015). In an ideal biotechnological conversion of lignocellulosic material into valuable products, this natural capacity should be mirrored in an industrial setup. However, by now, this has not been achieved in an economically reasonable degree. In a natural environment, the decomposition takes place over a prolonged period, often many years. This timescale is, however, not acceptable for an industrial process. Microbial production processes typically are performed in days-to-weeks time frame. A practical consequence of this requirement is that decomposition and biotechnological conversion of lignocellulose raw materials are performed in separate processes. Additionally, the fractionation into cellulosic, hemicellulosic and lignin fractions is usually performed (Chandel et al. 2018; de Bhowmick et al. 2018; Domínguez de María et al. 2015; FitzPatrick et al. 2010). Lignins have promising characteristics for innovative materials e.g. carbon fibers for light weight cars or as sources for aromatic polymers (Baker, D. A., Sedin, M., Landmer, A., Friman, L., Echardt, L 2017; Ragauskas et al. 2014; Upton and Kasko 2016). There are many established uses for the cellulosic fractions, e.g., microcrystalline cellulose for food applications, cellulosic fibers for paper, as raw material for bioplastic and saccharification by chemical or enzymatic hydrolysis to glucose and subsequent bioconversion. Hemicelluloses remains mostly unused although it is the second most abundant type of polysaccharides in nature and approximately 60 billion tons of hemicelluloses are produced annually (Gatenholm and Tenkanen 2003; Shahzadi et al. 2014). Hemicelluloses consist of valuable monosaccharides, including xylose, mannose, arabinose and galactose. In order to utilize the total potential of lignocellulose sugars, including hemicellulose sugars, increased requirements are therefore placed on process control, such as consumption of complex sugar mixtures in contrast to single sugar solutions, as well as accumulation of inhibitors. Another key element for efficient processes is the choice of suitable microorganisms. An ideal biocatalyst for a lignocellulose biorefinery should have high resistance to inhibitors and a large portfolio of enzymes to decompose and to metabolize all lignocellulose sugars. Some industrially relevant bacteria strains such as *Pseudomonas* are not able to use hemicellulose sugars as

carbon source or to depolymerize cellulose and hemicelluloses. In this case, strain engineering techniques are required.

1.1 Lignocellulosic biomass

Lignocellulose is a complex heteropolymer consisting of cellulose, hemicelluloses and lignins as well as small amounts of pectins, proteins and ash. It forms the structural framework of woody plant cell walls and its structure varies between different plants (Hendriks and Zeeman 2009; Jørgensen et al. 2007).

Of the polymer fractions in lignocellulose, cellulose is the main fraction, consisting of glucoses linked by β-1,4 glucosidic bonds. The long, linear, unbranched glucose chains are combined by hydrogen bonds into microfibrils. There are crystalline (organized) and amorphous (not well-organized) parts in cellulose. The crystalline structures are highly resistant and difficult to degrade for microorganisms (Ding and Himmel 2006; Eriksson and Bermek 2009; Hendriks and Zeeman 2009; Jørgensen et al. 2007). The cellulose microfibrils are embedded in a network of hemicelluloses, pectins and lignins (Eriksson and Bermek 2009). The total dry weight of hardwood, softwood and grasses (poaceae) contains approximately, 45±2%, 42±2%, 25-40% cellulose, respectively. Cotton, with a content of 80-95%, consist almost exclusively of cellulose (Saini et al. 2015).

Hemicelluloses are a group of heterogeneous plant cell wall polysaccharides. Unlike cellulose, the structures of hemicelluloses differ from plant to plant consisting mainly of the sugars D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, as well as, sugar acids such as D-glucuronic acid and 4-O-methyl-D-glucuronic acid. The monosaccharides of the backbones of hemicelluloses are linked via β-1,4 and β-1,3 glucosidic bonds (Ebringerová 2005; Jørgensen et al. 2007; Scheller and Ulvskov 2010). Usually hemicelluloses are divided into four groups according to their main sugars: xyloglucans, xylans, mannans and glucomannans, and β-(1→3,1→4)-glucans. Xyloglucans are the main hemicelluloses in the primary cell wall of dicotyledons. Glucoronoxylans dominate the secondary cell wall of dicotyledons and glucoronoarabinoxylans the primary and secondary cell wall of grasses. Glucomannans are present as minor components in primary and secondary cell wall of dicotyledons, while galactoglucomannans are the main hemicelluloses in the secondary cell wall of conifers. β-(1→3,1→4)-glucans are mainly found in Poales (Scheller and Ulvskov

2010). Consequently, D-mannose is the main hemicellulose monosaccharide in softwood, for example, spruce and pine. In hardwood (i.e., birch, poplar and oak) and poaceae, which accumulate in large amounts from agricultural waste, such as corn stover and straw, xylose is the principal hemicelluloses monosaccharide (Jørgensen et al. 2007). Hemicellulose chains are shorter and more branched than cellulose chains (Kuhad et al. 1997) and they are more thermosensitive in comparison to cellulose and lignins (LeVan et al. 1990; Winandy 1995). Hemicelluloses connect lignins with cellulose fibrils, thus giving lignocellulose a higher rigidity (Laureano-Perez et al. 2005).

Lignins are the most abundant non-polysaccharide fraction in lignocellulose. Hardwood and softwood have a typical lignin content of 20-25% and 28-32% (total dry weight) respectively (Eriksson and Bermek 2009). The lignin content of grasses is typically between 10-30% (Saini et al. 2015). Some herbaceous plants such as cotton (0-1.6%) or flax (2-5%) have a lower lignin content (Müssig et al. 2010). Lignins are a group of complex amorphous heteropolymers, consisting of three phenylpropane units, namely, para-coumaryl, coniferyl and sinapyl alcohols (Eriksson and Bermek 2009). The compressive strength of lignins give plants the strength to stand vertically. For this reason, tall plants such as trees have a high lignin content, whereas mosses or water plants have a low lignin content. Moreover, lignins are important for the impermeability and for the resistance against microbial attack and oxidative stress of plants (Eriksson and Bermek 2009).

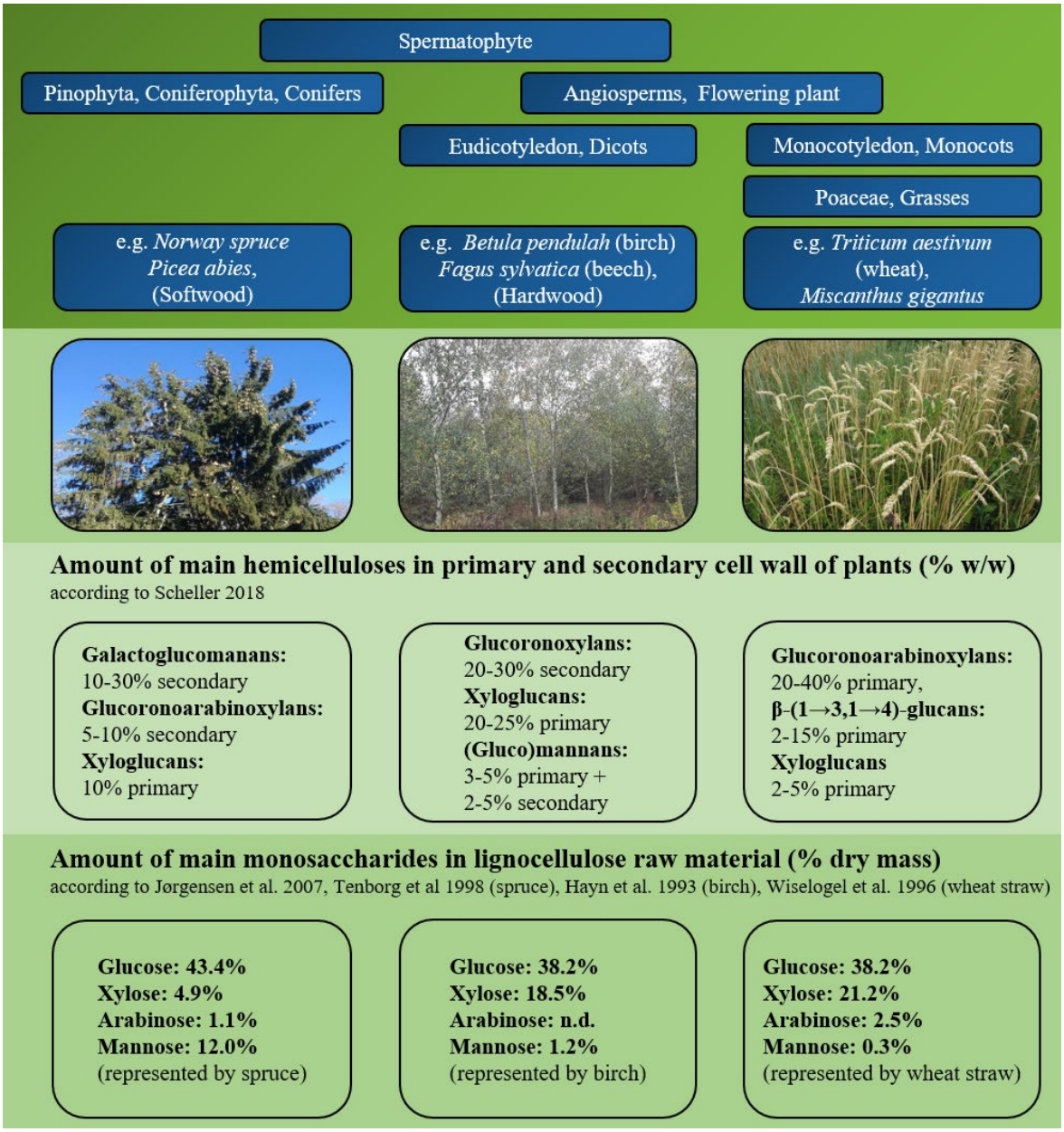

Figure 1: Sugar composition of common lignocellulosic materials in plants

Lignocellulose is the most abundant renewable raw material on earth and is available in large quantities as a cheap waste material from the food, agriculture, wood and paper industries and as municipal solid waste (van Dyk et al. 2013). The composition of these lignocellulosic materials varies strongly (Table 1). Furthermore, the reported compositions of the same materials often differ due to several reasons such as harvest time, harvest area, or differences in applied processes (van Dyk et al. 2013). Accordingly, data from Table 1 are to be regarded as a guide and not as absolute values. During the processing of potatoes, approximately

15-40% is lost as peeling (van Dyk et al. 2013) making an approx. annual production of 388 million tons (Mt) in 2017 (FAO 2019), which would be approx. 58–155 Mt of waste. Another potential source of lignocellulose are sugar beets with a global production of 301 Mt in 2017 (FAO 2019). When processing 1 t of sugar beets, approx. 70 kg exhausted dried pulp or 250 kg exhausted pressed pulp remains (van Dyk et al. 2013). Sarkar and coworkers reported the potential available amount of major agricultural wastes: wheat straw 354 Mt, rice straw 731 Mt and corn straw 203 Mt (Sarkar et al. 2012). For more detail information refer to following articles on agricultural waste (Reddy and Yang 2005; Saini et al. 2015; Sarkar et al. 2012) and on food residues (van Dyk et al. 2013).

Table 1: Composition of common lignocellulosic waste materials and residues

Material	Cellulose [%]	Hemi celluloses [%]	Lignins [%]	References
Wood residues				
Hardwood (general)	45±2	30±5	20±4	(Saini et al. 2015)
Softwood (general)	42±2	27±2	28±3	(Saini et al. 2015)
Hardwood barks	22-40	20-38	30-55	(Saini et al. 2015)
Softwood barks	18-38	15-33	30-60	(Saini et al. 2015)
Grasses (poaceae)				
Grasses (average)	25-40	25-50	10-30	(Howard et al. 2003)
Agricultural residues				
Wheat straw	37-41	27-32	13-15	(Reddy and Yang 2005)
Rice straw	28-36	23-28	12-14	(Reddy and Yang 2005)
Corn stover	38-40	28	7-21	(Saini et al. 2015)
Food industry residues				
Potato pulp	-	66	-	(van Dyk et al. 2013)
Sugar beet pulp	21-30	24-32	2-4	(van Dyk et al. 2013)
Carrot pomace	52	12	32	(Nawirska and Kwaśniewska 2005)
Bagasse	33-43	30	12-24	(van Dyk and Pletschke 2012)
Waste paper				
Newspaper	40-55	25-40	18-30	(Lee 1997)
Municipal solid wastes				
Primary waste water solids	10	-	26	(Cheung and Anderson 1997)

1.2 Hydrolysis

Lignocellulosic biomass is commonly decomposed before it is applied as a carbon source in biotechnological processes. In principle there are two different possibilities. Firstly, lignocellulose can be decomposed thermochemically. In this case the raw material can be gasified or decomposed by pyrolysis. The main product is a complex mixture containing not only the sugar fraction, but also unknown and toxic compounds. Even though the processes are not complicated, the further refinement of the decomposition products is commonly a challenge. Hydrolysis with chemicals or enzymes is the second decomposition possibility for lignocellulose. In general, hydrolysis is more complex than thermochemical processes, but the sugars so obtained are typically of high purity, and lignin can be separated comparably easily.

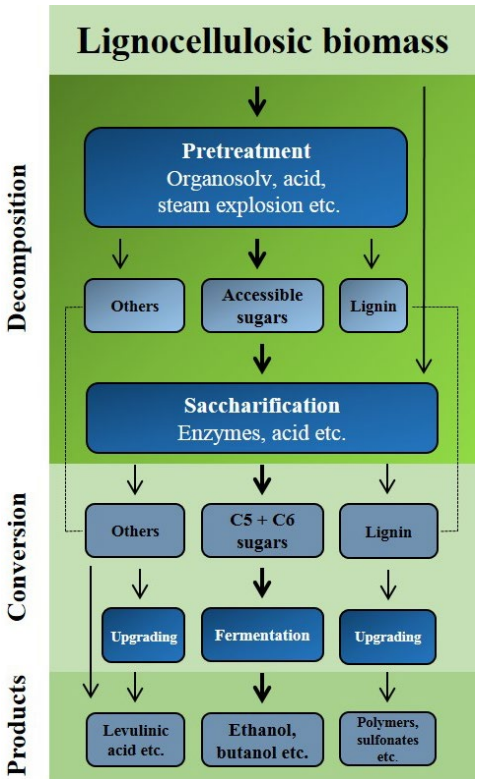

Figure 2: Overview of hydrolysis and conversion process of lignocellulosic biomass: decomposition, conversion and potential products

1.2.1 Pretreatment

There are many material characteristics of lignocellulose which limit hydrolysis, for example, the lignin content, degree of polymerization, cellulose crystallinity, porosity, feedstock particle size and cell wall thickness. Therefore, pretreatment techniques are frequently used prior to hydrolysis. Due to the high heterogeneity of lignocellulose and different hydrolysis processes there are numerous pretreatment methods, not just one ideal method. First, there are biological methods. Commonly lignocellulose is treated with white, brown and soft rot fungi. Second, physical methods including chipping, grinding or milling are applied for reducing particle size and depolymerization degree of lignocellulose material. Third, chemical pretreatment methods with bases like sodium hydroxide or with diluted/concentrated acids, such as sulfuric acid, are often used. Another chemical method is the organosolv process. Organic or aqueous solvents like glycol and ethanol are applied for solving lignin and hemicelluloses. The fourth group of pretreatments are physiochemical methods including steam explosion. Lignocellulose fibers are broken up by hot steam (180-240°C) and high pressure (1-3.5 MPa) phases followed by sudden pressure release (Alvira et al. 2010; Hendriks and Zeeman 2009). The organosolv process has the advantages that the saccharification rate is high, lignins and hemicelluloses are hydrolyzed, and it is applicable to a variety of raw materials. On the other hand, many inhibitors and by-products are generated and the solvents have to be recycled. The alkaline pretreatment also achieves high saccharification rates and removes the lignin and hemicellulose fractions. Disadvantages are the long processing time and the formation of non-convertible salts. Steam explosion is another pretreatment for enzymatic cleavage that achieves high sugar yields. The content of formed inhibitors can be high and the destruction of the lignin-carbohydrate matrix incomplete. Biological pretreatment methods are usually mild and environmentally friendly and only a few inhibitors and by-products are formed. However, the applications remain challenging as the process is very slow and involves careful monitoring of growth conditions, and lignoyltic microbial biocatalysts usually consume hemicelluloses and cellulose in addition to lignins (Menon and Rao 2012). Many research groups have focused on developing new pretreatment strategies, which have been reviewed extensively in the literature (Alvira et al. 2010; Hendriks and Zeeman 2009; Mosier et al. 2005; Taherzadeh and Karimi 2008). Recently, trends and new patents in the field of fractionation and pretreatment of lignocellulose were summarized (Domínguez de María et al. 2015). For the selection of a suitable pretreatment method, the following factors should be considered:

sugar yields; formation of toxic compounds; lignin recovery; energy requirements; cost and time efficiency (Yang and Wyman 2008). The purity or content of toxic substances, such as organic acids, furan aldehydes or phenols of the hydrolyzates, is one main indicator for applying them as carbon sources for biotechnological processes. The selection of microorganisms and the process design would be severely restricted in the case of highly contaminated hydrolyzates. Growth-limiting inhibitor concentrations can easily occur in fed-batch processes with high feed rates.

1.2.2 Enzymatic hydrolysis

Using nature as a model, enzyme mixes were designed for industrial hydrolysis. On the one hand, individually adapted enzyme cocktails are applied, while on the other hand, commercially-produced enzyme mixes from companies such as Novozyme or Genencor are used. An overview of several enzyme mixes is given (van Dyk and Pletschke 2012). In the following, the hydrolysis of cellulose and hemicellulose is presented. For more information on hydrolysis of lignins refer to (Janusz et al. 2017; Pollegioni et al. 2015)

Cellulose and hemicelluloses are mainly degraded by glycoside hydrolases (GHs). Enzyme groups, such as carbohydrate esterases (CEs) and polysaccharide lyases (PLs) are usually also involved. It is assumed that cellulase activity is mediated by three enzyme types. Endo1,4-β-glucanase (EC 3.2.1.4) attacks the cellulose chains within the amorphous structure and thereby generates many fragments. Cellulose 1,4-β-cellobiosidase (non-reducing end) (EC 3.2.1.91) and cellulose 1,4-β-cellobiosidase (reducing end) (EC 3.2.1.176) attack cellulose from the ends of the chains leading in the realise of tri- and disaccharides. The β-glucosidase (EC 3.2.1.21) hydrolyse the tri- and disaccharides to glucose. Due to the more complex structure of hemicelluloses, a greater variety of enzymes is required to degrade these polymers than for cellulose. In general, there are enzymes for depolymerization of the backbone and enzymes for removing the substituents (van Dyk and Pletschke 2012). Hemicellulases are glycosidases GHs, CEs and PLs. The following is focused on the degradation of the main hemicelluloses of softwood mannans and the main hemicelluloses in hardwood xylans. In the first step of mannan degradation, the backbone of the polymer is divided into mannotrioses and manndioses, which are catalyzed by the mannan endo-1,4-β-mannosidase (EC 3.2.1.78) (Ghosh et al. 2013; Gübitz et al. 1996; Shallom and Shoham 2003). Subsequently, the mannan pieces are hydrolyzed at the

non-reducing end in the presence of the exoenzyme β-mannosidase (EC 3.2.1.25) (Moreira and Filho 2008; van Zyl et al. 2010). Glucose is cleaved by β-glucosidases. These exoenzymes are usually only active against oligosaccharides, as most β-glucosidases are not able to cleave undivided long-chain mannans (Chauve et al. 2010; Xiao et al. 2004). Galactose residues are hydrolyzed by α-galactosidase (EC 3.2.1.22) (Jindou et al. 2002). Xylans are first depolymerized by the endo-1,4-β-xylanase (EC 3.2.1.8). Thereupon, the xylan 1,4-β-xylosidase (EC 3.2.1.37) hydrolyses the resulting oligosaccharides to xylose monomers. Besides, hemicelluloses have a lot of substituents such as galactose, arabinose and acetyl which are cleaved by enzymes such as non-reducing end α-L-arabinofuranosidase (EC 3.2.1.55) and α-galactosidase (EC 3.2.1.22). In addition to hydrolases, esterases are also involved in the degradation of hemicelluloses (Moreira and Filho 2016; van Dyk and Pletschke 2012).

1.2.3 Acid-based hydrolysis

In principle, lignocellulosic raw materials are treated with acid for a specific time and at a specific temperature. Sulphuric acid is most commonly used, but other acids such as hydrochloric acid are also common. It is possible to distinguish between the processes whether the acid is diluted at high temperature (approx. 200°C) or concentrated (30-70%) at low temperature (approx. 40°C). In the following the basic of both methods is presented. A comprehensive review of acid-based hydrolysis is given by (Taherzadeh and Karimi 2007).

Treatment of lignocellulose with concentrated acid has a long history. As long ago 1819, Braconnot performed experiments with cellulose and acids (Braconnot 1819; Taherzadeh and Karimi 2007). In the last decade, researchers pretreated lignocellulosic materials with 70% sulfuric acid followed by hydrolysis, which was initiated by adding water (van Groenestijn et al. 2008). Concentrated acid has the advantage over diluted acid since the process temperatures are lower and higher sugar yields are achieved. However, high material requirements due to the high acid concentration pose a considerable challenge for process design. As a consequence, materials such as ceramics or carbon are appropriate, but the acquisition costs would then be higher. In addition, efficient acid recovery is required to avoid high energy and acid consumption (Jones and Semrau 1984; Kumar and Wyman 2009; Taherzadeh and Karimi 2007). One current example is a hydrolysis process with high concentrated hydrochloric acid from the Green Sugar AG. Cellulose and hemicellulose

fraction are obtained in a two-step acid hydrolysis. The acid is then recovered by evaporation and lignin is used as an energy source for the process (Green Sugar AG 2018). For chemical hydrolysis, diluted acid with high temperature is often the method of choice. In this case the formation of inhibitors is critical due to the high temperatures required (Taherzadeh et al. 1997). For this reason, two- or multi-stage processes were developed. In the first part of the process, lignocellulose is treated at lower temperatures under milder conditions, resulting in the digestion of hemicelluloses. In the second part, the more stable cellulose is treated under higher temperatures (Harris et al. 1985).

1.3 Simultaneous saccharification and fermentation and consolidated bioprocessing

Besides processes in which decomposition and saccharification are carried out separately, lignocellulosic biomass can also be used directly as a carbon source for biotechnological processes. One possibility is simultaneous saccharification and fermentation (SSF), thus saccharification and fermentation are carried out in a single vessel, but the enzymes are produced separately. In the case of consolidated bioprocessing (CBP), all three processes (enzyme production, saccharification and fermentation) are carried out in a single step. SSF and CBP enable a decrease in equipment and material consumption and a reduction in process time. Since the sugars are metabolized directly, the risk of contamination is lower and the inhibitory effect of released sugars on hemicellulases and cellulases is largely avoided (Hasunuma and Kondo 2012; Olson et al. 2012; Salehi Jouzani and Taherzadeh 2015). A major challenge when using SSF is the difference in the respective temperature optima. Normally, the preferred temperature for saccharification is 50°C, but for most industrial relevant microorganisms the temperature should be between 28-38°C (Hari Krishna et al. 2001; Hasunuma and Kondo 2012; Jørgensen et al. 2007). In this context, research groups are working with temperature insensitive yeast strains for ethanol production (Abdel-Banat et al. 2010). CBP-compatible microbes need to be able to degrade lignocellulose simultaneously and to achieve high product titers. In light of the fact that such microorganisms do not occur naturally in nature, strain engineering is required to produce them (summarized by Olson et al. 2012).

1.4 Inhibitors and counter strategies

During pretreatment and hydrolysis of lignocellulose, toxic compounds are formed, specifically, pentoses are dehydrated to furfural and 2-furoic acid, and hexoses to hydroxymethylfurfural (HMF). Furans can further be degraded to formic acid, whereas HMF can be degraded to levulinic acid and formic acid under severe process conditions. Acetic acid is derived from acetic groups of hemicelluloses and phenolic compounds such as coniferyl aldehyde or ferulic acid are the result of hydrolysis of lignins. The formation of such inhibitors is dependent on the applied process, for example, acid-treated samples contain by-products including furans, aliphatic acids and phenolic compounds. Chemical pulping, including the organosolv process, causes the formation of aliphatic acids (Jönsson and Martín 2016). Normally, these toxic compounds are less concentrated in hydrolyzates than in other decomposed samples, including pyrolysis oil. Nevertheless the toxic compounds could have a negative impact on the process (Arnold et al. 2017; Jönsson et al. 2013; Palmqvist and Hahn-Hägerdal 2000a, 2000b). In addition, products such as ethanol and butanol or chemical additives, and solvents introduced during pretreatment may have an inhibitory effect. Challenging process conditions in the case of pH values, temperature and salt concentration may be also considered. This can be countered by less recalcitrant feedstock and mild pretreatment conditions, appropriate bioreactor setup or by applying additional detoxification steps, such as heating, vaporization, extraction, microbial and enzymatic treatment or adding chemical additives (Chandel et al. 2011; Jönsson et al. 2013; Jönsson and Martín 2016; Palmqvist and Hahn-Hägerdal 2000a, 2000b). Furthermore, employing microorganisms such as *P. putida* KT2440, which are insensitive to organic acids (Martins dos Santos et al. 2004) and aromatic compounds (Jimenez et al. 2002) are a viable possibility. Even when using insensitive microorganisms, problems regarding inhibitory effects may occur if processes with increased productivity are to be developed, for example, fed-batch processes. The concentrations of substances present in the substrates increase due to subsequent feeding, potentially reaching concentrations at which toxicity occurs. This effect may be countered by employing organisms which degrade rather than tolerate inhibitory compounds, or by using suitable process control strategy, such as a continuous process with removal of culture broth or in-situ filtration or dialysis methods.

Another strategy for coping with demanding substrates and process conditions is tolerance engineering. This is usually a challenging task, since a large number of inhibitors have to be controlled at the same time, the toxicity mechanisms of the inhibitors can be varying and often the exact inhibitor profile of a substrate is not known. One way is to change specific enzymes or pathways (Chen and Dou 2016; Mukhopadhyay 2015). For example, though genomic integration of hmf genes cluster, the catabolic pathway of these aldehydes in *P. putida* KT2440 was closed. The strain showed a higher resistance to HMF and was able to use it to some extent as a carbon source (Guarnieri et al. 2017). The furfural tolerance of an engineered isobutanol-producing *Escherichia coli* strain could be enhanced by increasing intracellular NAD(P)H pool via overexpression of NAD salvage pathway enzymes (Song et al. 2017). In *E. coli*, furfural is commonly reduced to less toxic furfuryl alcohol by alcohol dehydrogenase involving the consumption of NAD(P)H (Seo et al. 2016). Another approach is global tolerance engineering, which focuses on genes involved in more than one pathway, such as global regulator proteins, transcription factors, or chemical elicitors to enhance the stress tolerance of strains over a broader range (Chen and Dou 2016). By engineering of a global regulator cAMP receptor protein (CRP), *E. coli* became more stable against inhibitors, such as ethanol, acetate, butanol and process parameters such as low pH, oxidative and osmotic stress (Geng and Jiang 2015). New system biology techniques and screening methods can be powerful tools for tolerance engineering. In addition to the targeted alteration of certain metabolic pathways, laboratory evolution is also a frequently used method for production of resistant strains or analyzing stress mechanisms (Chen and Dou 2016).

1.5 Main characteristics of next generation lignocellulose biotechnology microorganisms

An ideal biocatalyst of a lignocellulose biorefinery should have the following characteristics (Figure 4). Since lignocellulosic raw materials have a highly compact and recalcitrant structure, most industrially relevant microorganisms cannot utilize them as a carbon source. Therefore, a depolymerization step is necessary to obtain easily metabolizable C5 and C6 sugars. In order to avoid this expensive and complex process step, microorganisms that can naturally degrade lignocellulose polymers are of interest. In addition, the use of the complete spectrum of lignocellulose sugars is desirable. It should be noted that besides glucose, other sugars are present in large amounts in plants. Softwoods, such as spruce and pine, have a mannose content of 12.0% and 11.7% of total dry weight, respectively. Xylose is the main sugar in hardwoods like birch with a xylose content of 18.5% of total dry weight and grasses with approximately the same amount of xylose (Jørgensen et al. 2007). Therefore, using strategies and knowledge from the field of synthetic biology might be promising. In this context, many research groups tried to complete and enhance the pentose metabolism of established microorganisms such as *Cornyebacterium glutamicum* (Kawaguchi et al. 2006; Kawaguchi et al. 2008; Radek et al. 2014), *P. putida* (Dvořák and de Lorenzo 2018; Meijnen et al. 2008; Wang et al. 2019) as well as *Saccharomyces cerevesia* (Kim et al. 2013).

Furthermore, inhibitory substances such as organic acids, acetate and furfural aldehydes can be formed/released during the degradation of lignocellulose. These inhibitors cause losses during the fermentation process and therefore robust microorganisms are preferred (Chandel et al. 2011; Jönsson et al. 2013; Jönsson and Martín 2016; Palmqvist and Hahn-Hägerdal 2000a, 2000b). In order to achieve efficient processes, strains with reduced formation of by-products such as sugar acids or extracellular polymers, should be chosen.

It is important that the selected microorganisms are suitable as production strains. Genetic modification techniques, such as introduction of foreign DNA, should be established. Furthermore, completely sequenced and annotated strains are ideal. Strains with good process parameters like specific productivity (qp), volumetric productivity (Pv), and yield coefficient ($Y_{P|S}$), together with sa flexible metabolism, are preferred. Ideally, the required precursors should already exist in sufficient amounts. In addition, it may be favorable to work with non-pathogenic strains due to security reasons, which require higher safety

precautions resulting in higher operational costs. Besides, the genetic stability of microorganims should be considered.

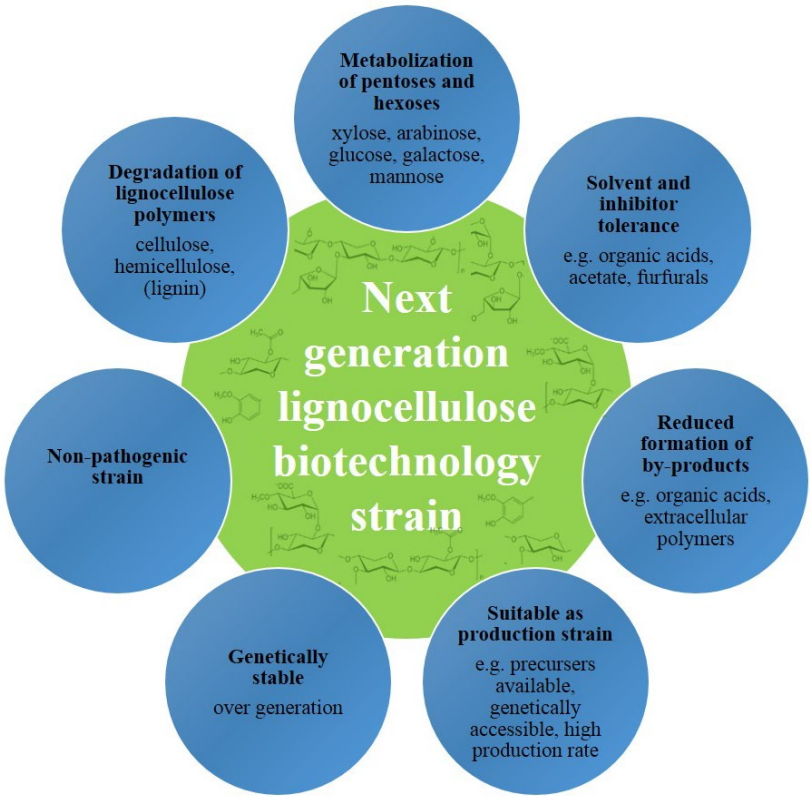

Figure 4: Main characteristics of the next generation lignocellulose biotechnology strains

1.6 *Pseudomonas putida* KT2440

The genus *Pseudomonas* was basically coined by the German scientist Walter Migula in 1894 and belongs to the class of γ-proteobacteria (Migula 1895; Palleroni 2010). *P. putida* KT2440 (Lurz et al. 1981) is a plasmid-free derivative of the strain *P. putida* mt-2 isolated from soil samples in Japan in 1960 (Nakazawa 2003). *P. putida* KT2440 is a Gram-negative, chemo-organotrophic and rod-shaped bacteria. Optimum growth conditions are at neutral pH-value and temperatures between 25°C and 30°C (Carter et al. 1995). *P. putida* KT2440 has a potential for biotechnological application due to its fast growth, robustness, simple nutritional requirements and versatile metabolism (Loeschcke and Thies 2015; Poblete-Castro et al. 2012; Timmis 2002). This strain has a comprehensive and robust metabolism

regarding organic acids (Martins dos Santos et al. 2004) and aromatic compounds (Jimenez et al. 2002). Compared to other microorganisms including *E. coli*, *P. putida* KT2440 shows a high stability towards common industrial chemicals such as toluene, ethanol and p-coumaric acid (Calero et al. 2018; Johnson and Beckham 2015; Nikel and Lorenzo 2014). It is completely sequenced (Nelson et al. 2002) and, unlike other *Pseudomonas* species, this strain is certificated as non-pathogenic in ATCC (American Type Culture Collection). Furthermore, promising experiments have been carried for the development of product-producing strains out namely, polyhydroxy-alkanoate (Davis et al. 2013) or rhamnolipids (Arnold et al. 2019; Beuker et al. 2016b; Cha et al. 2008; Wittgens et al. 2011; Wittgens et al. 2018).

However, as shown in chapter 1.8, the *P. putida* KT2440 enzyme profile is poor for the metabolism of lignocellulose sugars. It is not able to degrade lignocellulose polysaccharides and can only metabolize glucose, but not one single hemicellulose monosaccharide. Accordingly, strain engineering is required to produce *P. putida* KT2440 strains as hosts for lignocellulosic biorefinery.

1.7 Cellvibrio japonicus

Basically, there are two possible strategies for obtaining bacteria as biocatalysts for the lignocellulosic biorefinery. In the first case, an already established strain *P. putida* KT2440 is chosen because of reasons such as availability of precursors or robustness. An opposite approach would be the search for strains that are already naturally equipped with a large enzyme portfolio for the metabolism of lignocellulose mono- and/or polysaccharides like *C. japonicus* (Braithwaite et al. 1995; DeBoy et al. 2008; Gardner 2016; Gardner and Keating 2010, 2012).

The Gram-negative saprophytic bacterium *C. japonicus* strain UED107 was first isolated from field soil samples as part of a taxonomic study of *Pseudomonas* in Japan in 1948. It was initially classified as *Pseudomonas fluorescen*s subspecies *cellulosa* since *C. japonicus* showed similar properties to *P. fluorescens* and was able to degrade cellulose (Ueda et al. 1952). Subsequent biochemical and chemotaxonomic analysis showed little similarities to *P. fluorescens* according to the fatty acid profile and therefore a renaming to *P. cellulose* was recommended (Dees et al. 1995). The final reclassification into the genus *Cellvibrio* and

the associated renaming to *Cellvibrio japonicus* was a result of molecular biology investigations, including 16S rDNA sequence analysis (Humphry et al. 2003).

Many studies revealed that *C. japonicus* has an extensive portfolio of plant cell wall-degrading enzymes (DeBoy et al. 2008; Gardner 2016; Gilbert et al. 1987; Horlamus et al. 2019a; McKie et al. 1997). Genomic and bioinformatic analyses predicted that *C. japonicus* has about 130 glycoside hydrolases and a vast repertoire of glycoside lyases and esterases (DeBoy et al. 2008). The degradation mechanisms of cellulose and hemicellulases by *C. japonicus* were described and summarized in (Gardner 2016) and is described in detail in chapter 4.1. Furthermore, it was recently demonstrated that *C. japonicus* is able to metabolize the polysaccharide chitin (Forsberg et al. 2016). Due to these comprehensive metabolic capabilities, C. *japonicus* is a well-established candidate for the investigation of microbial plant cell wall breakdown (Gardner 2016; Gardner and Keating 2012). Furthermore, *C. japonicus* could be a promising host for the lignocellulose biorefinery. Previous studies demonstrated that this bacterium can be cultivated in the minimal media M9 and MOPS (morpholinopropane sulfonic acid) at 30°C and with cell wall polysaccharides as the sole carbon source (Gardner 2016; Gardner and Keating 2012). Moreover, genetic modifications have already been reported for this strain (Gardner and Keating 2010). Besides, small amounts of ethanol could be produced by applying the “broad hast range” plasmid pBBR1_MCS5 with the genes *pdc* and *adhB* encoding pyruvate decarboxylase and alcohol dehydrogenase, respectively (Gardner and Keating 2010). To date, there has been no other publication with *C. japonicus* as biocatalyst for production of rhamnolipids or other biosurfactants.

1.8 Degradation and metabolization of lignocellulose sugars by *Pseudomonas putida* KT2440 and *Cellvibrio japonicus*

Lignocellulose hydrolyzates solutions commenly contain not only monosaccharides, but also di-, oligo- and polysaccharides. Accordingly, it is necessary to consider the entire enzymatic system from degradation to metabolization of lignocellulose sugars in order to use them efficiently. In line with the theme of this work, the chapter "Metabolization of

monosaccharides" deals mainly with the metabolic pathways of *P. putida* KT2440 and the chapter "Degradation of polysaccharides" concentrates on *C. japonicus*.

1.8.1 Degradation of polysaccharides

In nature, the decomposition of lignocellulose is performed by complex enzyme systems, however, this has not yet been clarified in detail (Banerjee et al. 2010; Harris et al. 2010; Merino and Cherry 2007). Mostly white, brown and soft rot fungi and not bacteria are known as lignocellulose degraders (Sánchez 2009). An exception is the bacterium *C. japonicus*. This strain has an exceptionally high proportion of cellulases and hemicellulases. For that reason, *C. japonicus* is used as a model organism for elucidating the microbial degradation system of lignocellulose polysaccharides (Gardner and Keating 2012). According to the model of Gardner, a lytic polysaccharide mono-oxygenase possibly cleaves, with the help of redox proteins, the crystalline part of cellulose (Gardner 2016). Furthermore, one exo-acting cellobiohydrolase and several endoglucanases are responsible for cleaving cellulose chains in smaller pieces. The enzymes are secreted outside the cell since only oligo-, di- and monosaccharides are able to transport through the cell walls. A β-glucosidase is located in the periplasm and it is predicted that there are enzymes of this class that are supposed to be both inside and outside the cell. The model for degradation of (arabino)xylans includes several endoxylanases for depolymerizing xylan chains to smaller pieces. Acetyl esterases, feruloyl esterase and α-arabinofuranosidase remove acetyl acid and ferulic acid groups as well as arabinose sidechains, respectively. Glucuronic acid and arabinose sidechains are cleaved from xylan oligosaccharides by α-glucosidase and α-arabinofuranosidase located at the cell surface, respectively. The unbranched oligosaccharides are transported into the periplasm through the outer membrane where they are degraded by an endoxylanase. The released xylobiose is cleaved to xylose in the cytoplasm by a predicted β-xylosidase. The oligo- and monosaccharide transporters have not yet been identified in the case of xylans or cellulose (Gardner 2016).

P. putida KT2440 is not able to metabolize cellulose and hemicelluloses (Kanehisa and Goto 2000; Karp et al. 2017; Udaondo et al. 2018). Recently, a *P. putida* EM42 strain (genome genome-edited derivative of *P. putida* KT2440). for metabolizing cellobiose was constructed by using the genes *bglC* coding β-glucosidase (EC 3.2.1.21) from *Thermobifida fusca*. No additional transporter was required as cellobiose passed the inner

cell membrane through the glucose ATB-binding cassette (ABC) transporter of *P. putida* EM42 (Dvořák and de Lorenzo 2018).

1.8.2 Metabolization of monosaccharides

Unlike most microorganisms *Pseudomonas* does not possess 1-phosphofructokinase (EC 2.7.1.56) and accordingly uses the Entner-Doudoroff (ED) pathway and not the glycolysis (Embden-Meyerhof-Parnas pathway) for metabolization of carbohydrates. Glucose is metabolized to pyruvate over the intermediates 6-phosphoglucanolactone and 2-keto-3-deoxy-phosphoglucanate to pyruvate. Compared to glycolysis, organic acids can also be metabolized and one ATP and not two ATP are regenerated (Martins dos Santos et al. 2004; Nikel et al. 2015; Udaondo et al. 2018). Glucose is taken up into the cells by the phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS). A complex mechanism, including $EIIA^{Glu}$, adenylate cyclase, cyclic AMP (cAMP) and cAMP receptor protein (CRP), which inhibits the uptake of other sugars, such as D-xylose or L-arabinose when glucose is present (carbon catabolite repression) (Desai and Rao 2009; Postma et al. 1993). Glucose is transported to the outer membrane into the periplasm through porin OprB in *P. putida*. In the periplasm, glucose can be directly transported in the cytosol by the glucose ABC transporter or converted to gluconate or further to 2-ketogluconate and then transported in the cytosol via gluconate H^+symporter (GntP) and 2-ketogluconate H^+symporter (KguT), respectively (Martins dos Santos et al. 2004; Nikel et al. 2015; Udaondo et al. 2018).

In principle, there are four possible pathways to metabolize the pentose xylose: isomerase pathway, xylose reductase-xylitol dehydrogenase (XRDH) pathway, Weimberg and Dahms pathway (Karatzos et al. 2012; Kim and Woo 2018). Many bacteria, including *E. coli*, metabolize D-xylose via the isomerase pathway. Xylose is first isomerised to D-xylulose and then phosphorylated to D-xylulose 5-phosphate before it enters the pentose phosphate pathway (PPP) (Desai and Rao 2009). *P. putida* KT2440 is not able to metabolize xylose due to the lack of xylose isomerase (EC 5.3.1.5) and xylulokinase (EC 2.7.1.17) encoded by the genes *xylA* and *xylB*, respectively (Kanehisa and Goto 2000; Karp et al. 2017; Meijnen et al. 2008). However, by overexpression of the *xylAB* operon of *E. coli*, a xylose metabolizing strain could be developed (Meijnen et al. 2008). The XRDH pathway is typical for fungi. Two steps are required to metabolize D-xylose via intermediate xylitol to

D-xylulose (Jeffries 1983), while D-xylose is directly isomerized to D-xylulose in the isomerase pathway. In the Weimberg pathway D-xylose is metabolized to α-ketoglutarate via the intermediates D-xylonolactone, D-xylonate, 2-keto-3-deoxy-xylonate and α-ketoglutarate semialdehyde (Meijnen et al. 2009; Radek et al. 2014; Weimberg 1961). With the Weimberg pathway, no carbon dioxide is lost during the metabolism from D-xylose to α-ketoglutarate. This is an advantage over the isomerase pathway where 17% of the carbon is lost (Radek et al. 2014). A xylose metabolizing *Pseudomonas* strain could be engineered by knocking-in the *xylABCD* operon from *Caulobacte crescentus*. It could be shown, that only the *xylD* gene encoding D-xylonate dehydratase was essential and that *xylX and xylA* encoding 2-keto-3-deoxy-D-xylonate and α-ketoglutaric semialdehyde dehydrogenase, respectively, greatly improved growth. At the beginning of the pathway from D-xylose to D-xylonate D-glucose dehydrogenase has a clearly higher impact than the D-xylose dehydrogenase (encoded by *xylB*). This suggests that the first part of the WMB pathway, conversion from D-xylose to D-xylonate, takes place predominantly in periplasm (Meijnen et al. 2009). The Dahms pathway is the same as the Weimberg pathway up to the formation of the intermediate 2-keto-3-deoxy-xylonate. Then 2-keto-3-deoxy-xylonate is metabolized to pyruvate and glycolaldehyde (Kim and Woo 2018; Rossoni et al. 2018; Wang et al. 2018)

L-arabinose is metabolized to D-xylulose 5-phosphate by the enzymes L-arabinose isomerase (EC 5.3.1.4), ribulokinase (EC 2.7.1.16), L-ribulose-5-phosphate 4-epimerase (EC 5.1.3.4) encoded by the genes *araA*, *araB* and *araD* before it enters the PPP. *P. putida* KT2440 is not able to metabolize arabinose because it lacks all these enzymes (Kanehisa and Goto 2000; Karp et al. 2017). The *xylAB* and *araBAD* operons of *E. coli* are often used to engineer pentose metabolizing strains. In *E. coli* the arabinose system is regulated by the AraC protein and the uptake into the cells across the boundary membrane is carried out by the ABC transporter AraFGH and the H^+symporter: AraE. Xylose uptake system is regulated by the XylR and the xylose transporters XylFGH and XylE. Although both systems have a similar structure, arabinose is preferably transported through the membrane by the low-affinity H^+symporter, whereas xylose is transported by the high-affinity ABC transporter XylFGH. Furthermore, xylose can be taken up by the arabinose transporter set and arabinose can be taken up by the xylose transporter set. Moreover, xylose and arabinose are also taken up if both transporter systems are deleted (Desai and Rao 2009; Hernandez-Montalvo et al. 2001;

Kang et al. 1998; Khlebnikov et al. 2001). In *P. putida* it was suggested, that xylose mainly passes through the outer membrane via the OprB-1 porin and through the inner membrane via the glucose ABC transporter, as both corresponding genes were upregulated during xylose metabolization, and no growth could be analyzed after deletion of these genes (Meijnen et al. 2012). However, in mixed cultivation the pentose sugars are almost not metabolized in the presence of glucose (Horlamus et al. 2019b, Wang et al. 2019). Co-utilization of glucose and xylose was achieved by overexpression of the xylose H^+symporter encoded of *xylE* from *E. coli* in *P. putida* (Dvořák and de Lorenzo 2018). For co-utilization of arabinose, the overexpression of *araE* or *xylE* could be a solution.

The metabolism of mannose is carried out via an intermediate of glycolysis, the fructose 6-phosphate. A possible metabolic pathway is via the enzyms hexokinase (HK, EC 2.7.1.1) and mannose-6-phosphate isomerase (AlgA, EC 5.3.1.8). The hexokinase for the phosphorylation of mannose is missing in *P. putida* KT2440. In most organisms, galactose is catabolized through the Leloir pathway. Initially, aldose-1-epimerase (GalM, EC 5.1.3.3) catalyzes the reaction from β-D-galactose to α-D-galactose. Subsequently, α-D-galactose is phosphorylated via the galactokinase (EC 2.7.1.6) encoded by *galK*. Uridine diphosphate (UDP)-glucose-hexose-1-phosphate uridylyltransferase (GalT, EC 2.7.7.12) facilitate the transformation from galactose-1-phosphate to glucose-1-phosphate by using UDP-glucose as cofactor. The recovery from UDP-galactose to UDP-glucose is carried out with the help of the UDP-glucose 4-epimerase (GalE, EC 5.1.3.2). Glucose-1-phosphate is transformed to the glycolysis intermediate glucose-6-phosphate via the phosphoglucomutase (PgM, EC 5.4.2.2) (Bozell and Petersen 2010; Caputto and Leloir 1949; Frey 1996; Trucco et al. 1948; Wilkinson 1949). For metabolization of galactose via the Leloir pathway, *P. putida* KT2440 is lacking the genes *galK* and *galT* (Kanehisa and Goto 2000; Karp et al. 2017).

Similar to *P. putida* KT2440, *C. japonicus* lacks the enzyme 1-phosphofructokinase for metabolizing glucose via glycolysis. However, it has all the enzymes to metabolize glucose via the ED. *C. japonicus* is able to metabolize xylose (isomerase pathway) and arabinose. For metabolizing galactose through the Leloir pathway, *C. japonicus* is missing the *galT* genes. In the case of mannose this strain poses the fructokinase (EC 2.7.1.4), but misses the mannose isomerase (EC 2.7.1.56) (Kanehisa and Goto 2000; Karp et al. 2017). No information could be found about the transportation systems.

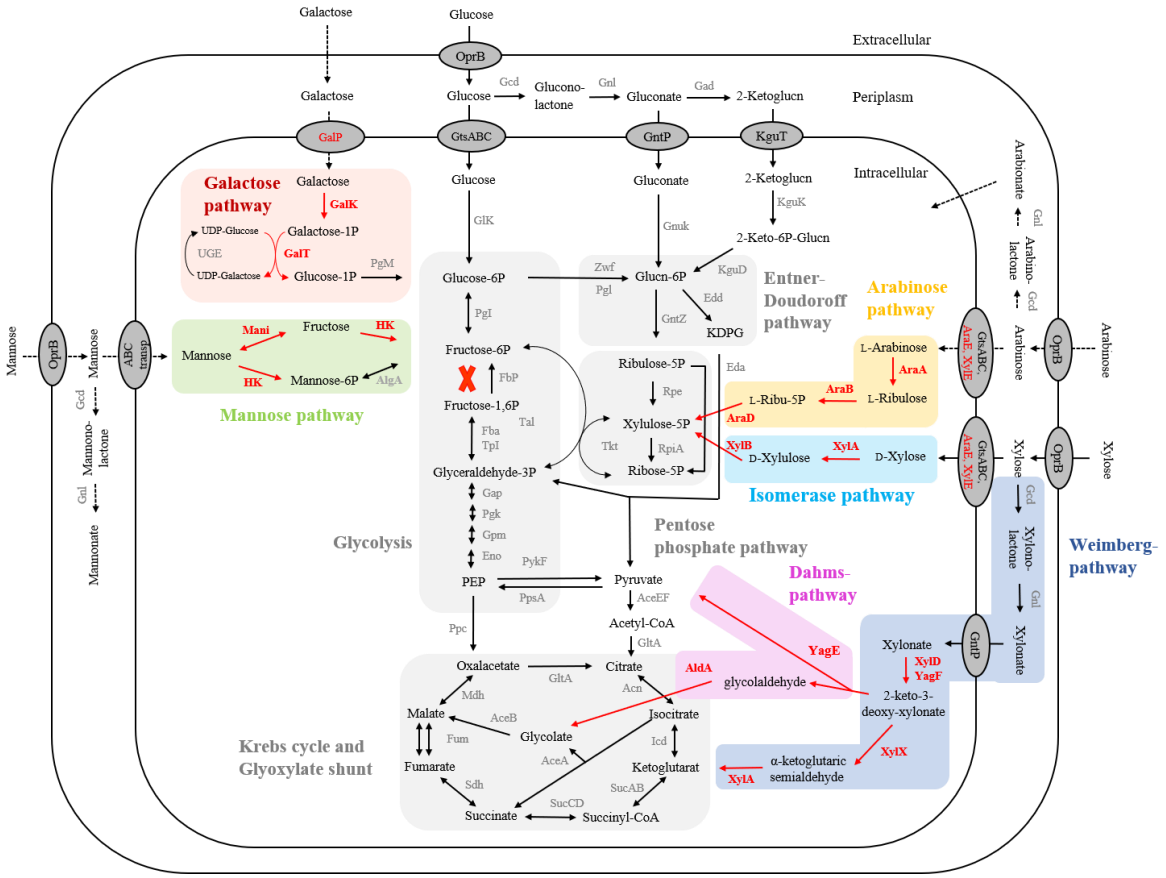

Figure 3: Simplified scheme of glucose metabolism and suggest pathways by genetic engineering for xylose, arabinose, mannose and galactose in *P. putida* KT2440. Abbrevation list of enzymes and transporters are listed in Annex 1. Glucose metabolism, glycolysis, pentose phosphate pathway, krebs cycle and glyoxylate shunt (Kanehisa and Goto 2000; Karp et al. 2017; Udaondo et al. 2018), Weimberg, Dahms and Isomerase pathway (Dvořák and de Lorenzo 2018, Kanehisa and Goto 2000; Karp et al. 2017; Kim and Woo 2018; Köhler et al. 2015; Meijnen et al. 2009; Rossoni et al. 2018; Weimberg 1961), arabinose and galactose pathway (Kanehisa and Goto 2000; Karp et al. 2017).

1.9 Aim of the thesis

The aim of this thesis was to develop and evaluate bacterial-based biotechnological processes capable of using hemicellulose sugars as a carbon source. The strains *P. putida* KT2440 and *C. japonicus* were used as microbial biocatalysts.

Since *P. putida* KT2440 is not able to metabolize xylose and arabinose, the first purpose of this project was to construct strains that can metabolize these two sugars. Furthermore, the growth characteristics of these strains on glucose, xylose and arabinose as single sugars and as mixtures were displayed. The second goal was to evaluate the potential of biotechnological conversion of lignocellulose hydrolyzates by *P. putida* KT2440. In this context different hydrolyzates were screened as a carbon source for this bacterium. Later, the inhibitory effect of major toxic substances in lignocellulose hydrolyzates on the growth of this strain were investigated. Last, initial feeding strategies for applying lignocellulose sugar mixtures as a carbon source in fed-batch bioreactor cultivations with *P. putida* KT2440 were developed. The third goal was to evaluate *C. japonicus* as a potential host strain for one-step bioconversion of xylans to rhamnolipids.

2 Manuscripts

Authors´ contributions to these publications

1. Wang Y, Horlamus F, Henkel M, Kovacic F, Schläfle S, Hausmann R, Wittgens A, Rosenau F, Growth of engineered *Pseudomonas putida* KT2440 on glucose, xylose and arabinose: Hemicellulose hydrolysates and their major sugars as sustainable carbon sources, Global Change Biology Bioenergy 2019, 11:249–259. doi: 10.1111/gcbb.12590

- **YW** and **FH** contributed equally to this work. **YW** and **FH** designed, planned, executed and experiments, collected and interpreted the data, created the graphs and drafted the manuscript. **MH** designed and planned the experiments. **KF** contributed to interpretation of the experiment. **SS** produced hydrolyzates and contributed to interpretation of the experiments. **AW** significantly contributed to conception and design of the study and interpretation of the experiments. **RH** and **FR** substantially contributed to conception and design of the conducted experiments. All authors read and approved the final version of the manuscript.

Place, Date

Signature of the supervisor

2. Horlamus F, Wang Y, Steinbach D, Vahidinasab M, Wittgens A, Rosenau F, Henkel M, Hausmann R, Potential of biotechnological conversion of lignocellulose hydrolyzates by *Pseudomonas putida* KT2440 as model organism for a bio-based economy, Global Change Biology Bioenergy 2019, 102:1254 2019. doi: 10.1111/gcbb.12647

   - **YW** and **FH** contributed equally to this work. **FH** designed, planned and executed the experiments, collected and interpreted data, created the graphs and drafted the manuscript. **YW** executed part of the bioreactor cultivations and contributed to interpretation of the experiment. **MV** executed part of the experiments and collected and evaluated corresponding data. **DS** produced hydrolyzates, performed quantitiave analysis of organic acids and furfural aldehydes in hydrolyzates and contributed to interpretation of the experiment. **AW** and **FR** contributed to interpretation of the experiment. **MH** significantly contributed to conception and design of the study and interpretation of the experiments. **RH** substantially contributed to conception and design of the conducted experiments. All authors read and approved the final version of the manuscript.

3. Horlamus F, Wittgens A, Noll P, Michler J, Müller I, Weggenmann F, Oellig C, Rosenau F, Henkel M, Hausmann R, One-step bioconversion of hemicellulose polymers to rhamnolipids with *Cellvibrio japonicus*: A proof-of-concept for a potential host strain in future Bioeconomy, Global Change Biology Bioenergy 2019, 11:260–268. doi: 10.1111/gcbb.12542

   - **FH** designed, planned and executed the experiments, collected and interpreted data, created the graphs and drafted the manuscript. **AW** and **FR** generated the plasmid pSynPro8oT and contributed to interpretation of the experiment. **NP**, **WF** executed adjustments for the HPTLC method. **MI** and **MJ** executed or performed part of the experiments and collected and evaluated corresponding data. **OC** helped to evaluated the mass spectrometric experiments. **MH** significantly contributed to conception and design of the study and interpretation of the experiments. **RH** substantially contributed to conception and design of the conducted experiments. All authors read and approved the final version of the manuscript.

Place, Date

Signature of the supervisor

2.1 1st Publication:
Growth of engineered *Pseudomonas putida* KT2440 on glucose, xylose, and arabinose: Hemicellulose hydrolysates and their major sugars as sustainable carbon sources

Wang Y*, Horlamus F*, Henkel M, Kovacic F, Schläfle S, Hausmann R, Wittgens A, Rosenau F (*The authors contributed equally to this work)

Global Change Biology Bioenergy 2019, 11:249–259

DOI: 10.1111/gcbb.12590

Lignocellulosic biomass is the most abundant bioresource on earth containing polymers consisting mainly 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 growth characteristics on various mixtures of these sugars and the possibility of using lignocellulosic hydrolysate as substrate for the recombinant strains were investigated.

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

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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.

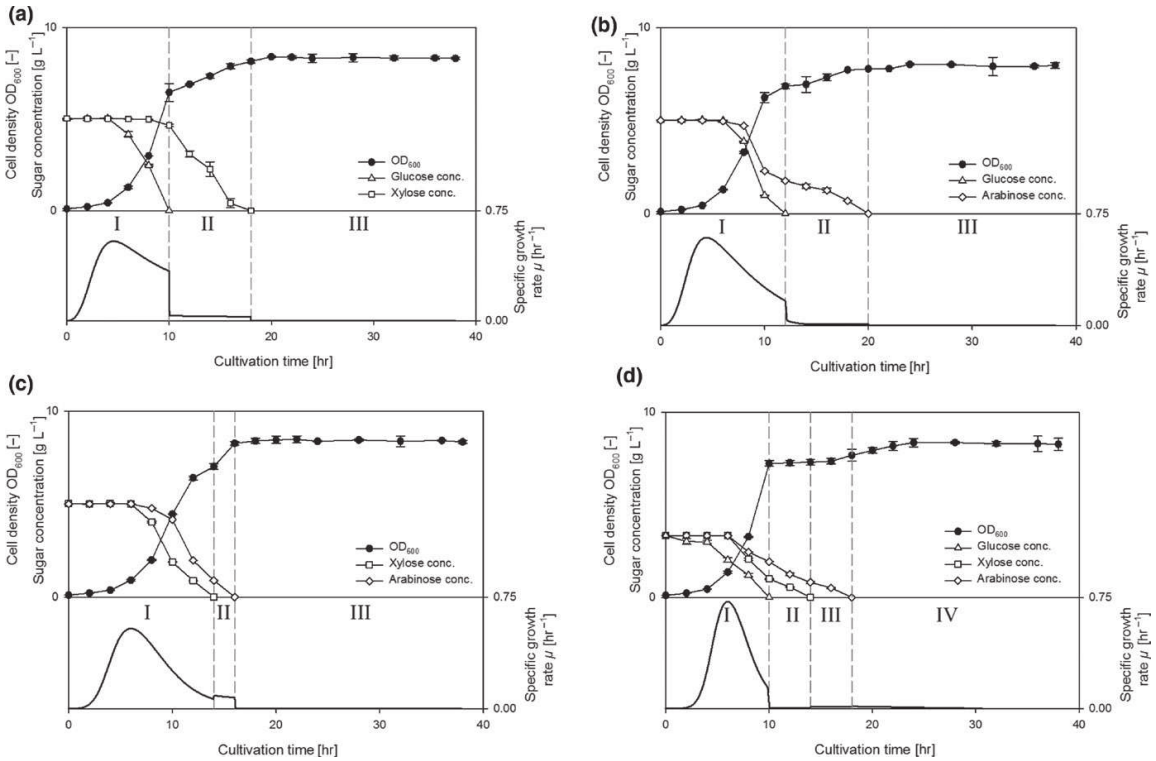

FIGURE 1 Growth performance of *Pseudomonas putida_xylAB* using mixtures of sugars as carbon source. The strain *P. putida* KT2440_*xylAB* (filled circles) was cultivated in Wilms-KPi medium containing mixtures of sugars with a total amount of 10 g/L equally distributed on glucose/xylose (a), glucose/arabinose (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

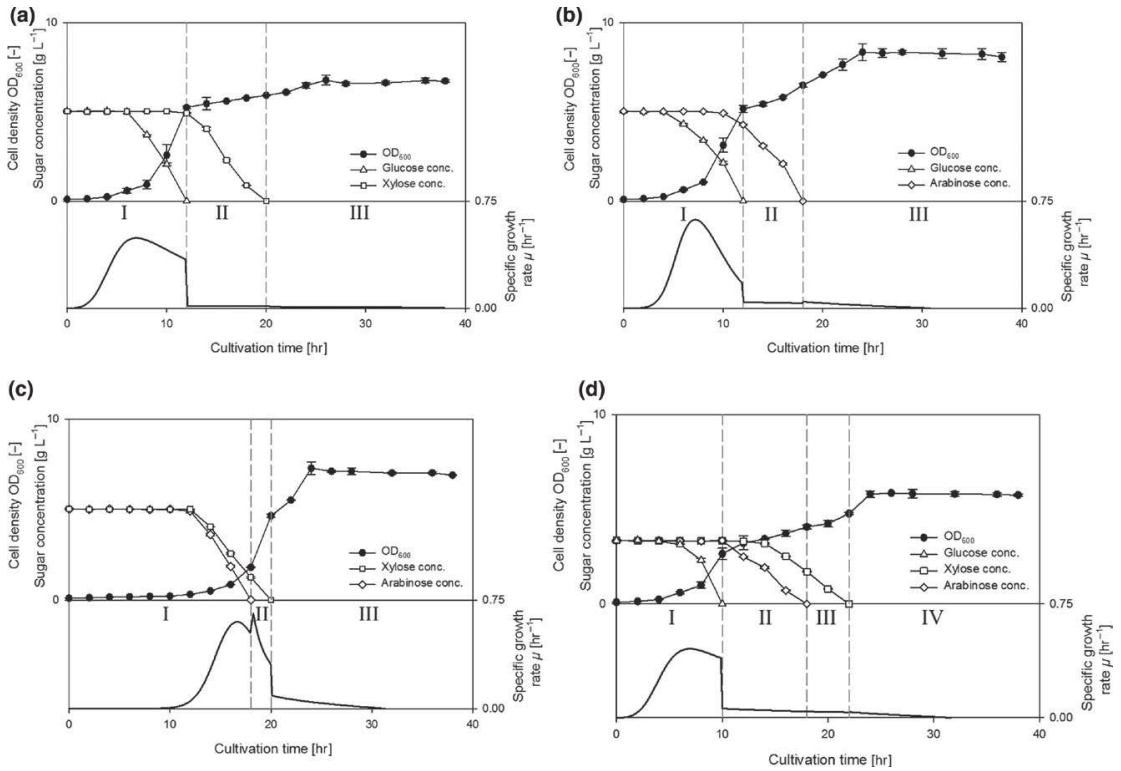

FIGURE 2 Growth performance of *Pseudomonas putida_araBAD* using mixtures of sugars as carbon source. The strain *P. putida* KT2440_*araBAD* (filled circles) was cultivated in Wilms-KPi medium containing mixtures of sugars with a total amount of 10 g/L equally distributed on glucose/xylose (a), glucose/arabinose (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.

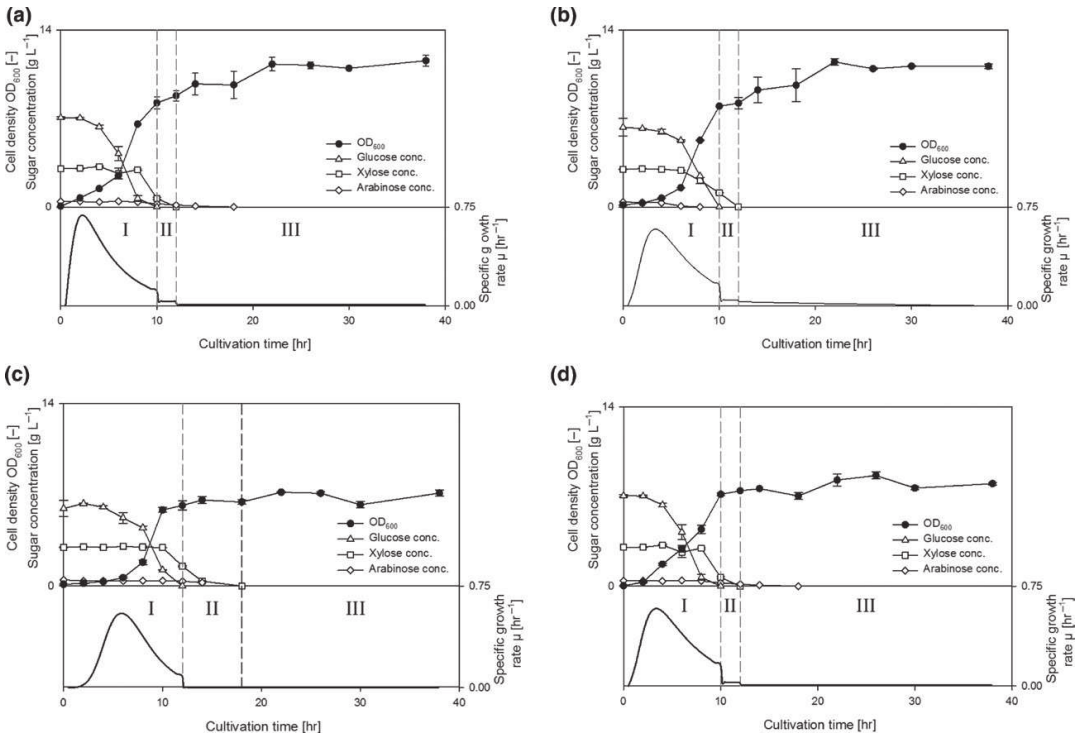

FIGURE 3 Growth performance of recombinant *Pseudomonas putida* using wheat straw hydrolysate as carbon source. The strains *P. putida* KT2440_*xylAB* (a, b) and *P. putida* KT2440_*araBAD* (c, d) (filled circles) were cultivated in Wilms-KPi medium containing real wheat straw hydrolysate (a, c) or artificial 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 (μ_{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.

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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

2.2 2nd Publication:
Potential of biotechnological conversion of lignocellulose hydrolyzates by *Pseudomonas putida* KT2440 as model organism for a bio-based economy

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Global Change Biology Bioenergy 2019, 11:1421-1434

DOI: 10.1111/gcbb.12647

Lignocellulose-derived hydrolyzates typically display a high degree of variation depending on applied biomass source material as well as process conditions. In this study, we investigate the potential of biotechnological conversion of lignocellulose hydrolyzates by *P. putida* KT2440 and its suitability as a model organism.

Received: 24 June 2019 | Accepted: 26 August 2019

DOI: 10.1111/gcbb.12647

ORIGINAL RESEARCH

GLOBAL CHANGE BIOLOGY BIOENERGY

WILEY

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

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Funding information
Ministry of Science, Research and the Arts of Baden-Württemberg, Grant/Award Number: 7533-10-5-86A and 7533-10-5-86B

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.

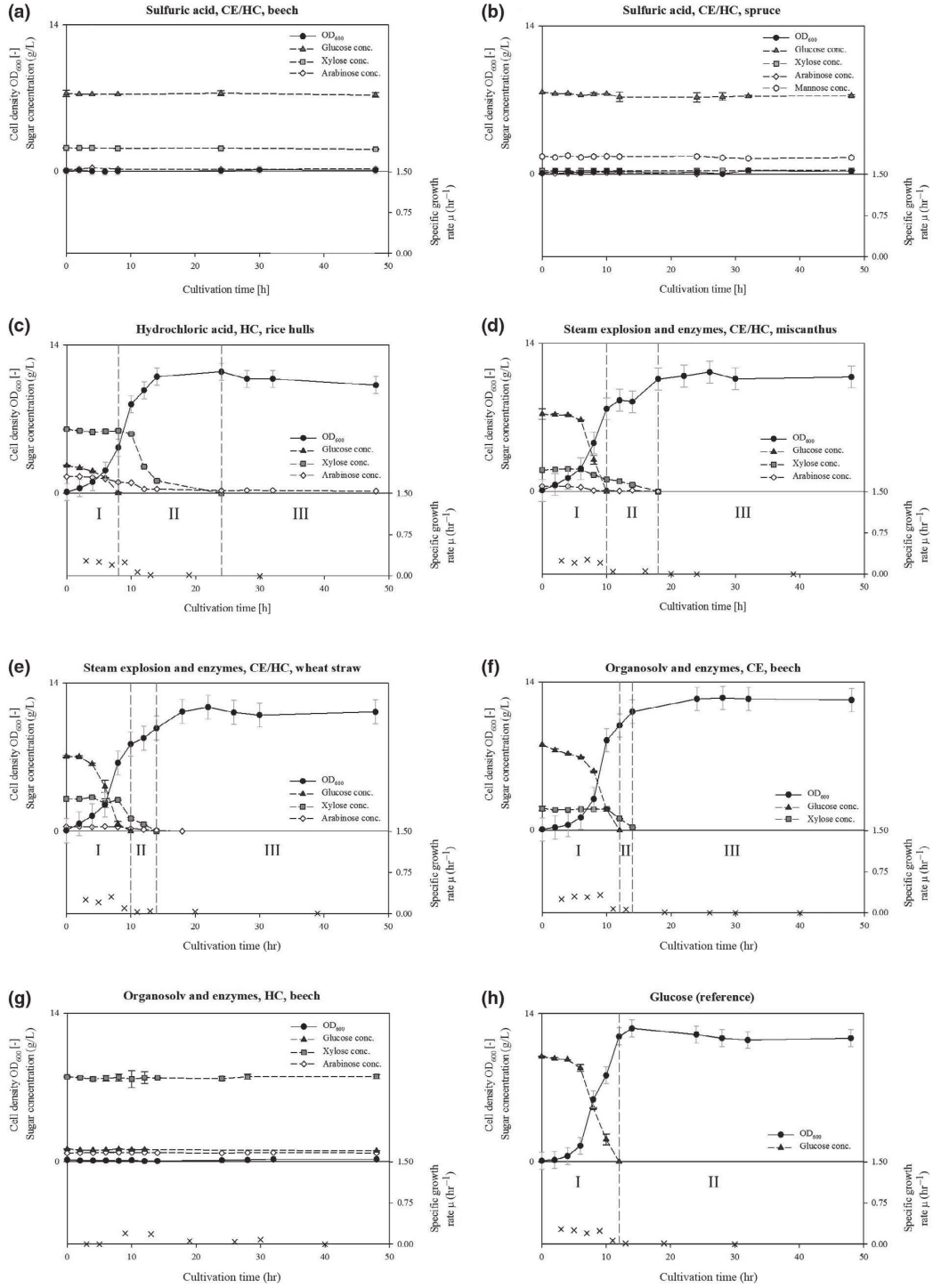

FIGURE 1 Time course of cultivation of *Pseudomonas putida* KT2440_*xylAB* with different lignocellulose hydrolyzates as carbon source. The specific growth rates are separately shown in the lower diagrams and single growth phases are indicated with roman numbers: phase I, mainly consumption of the first 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,

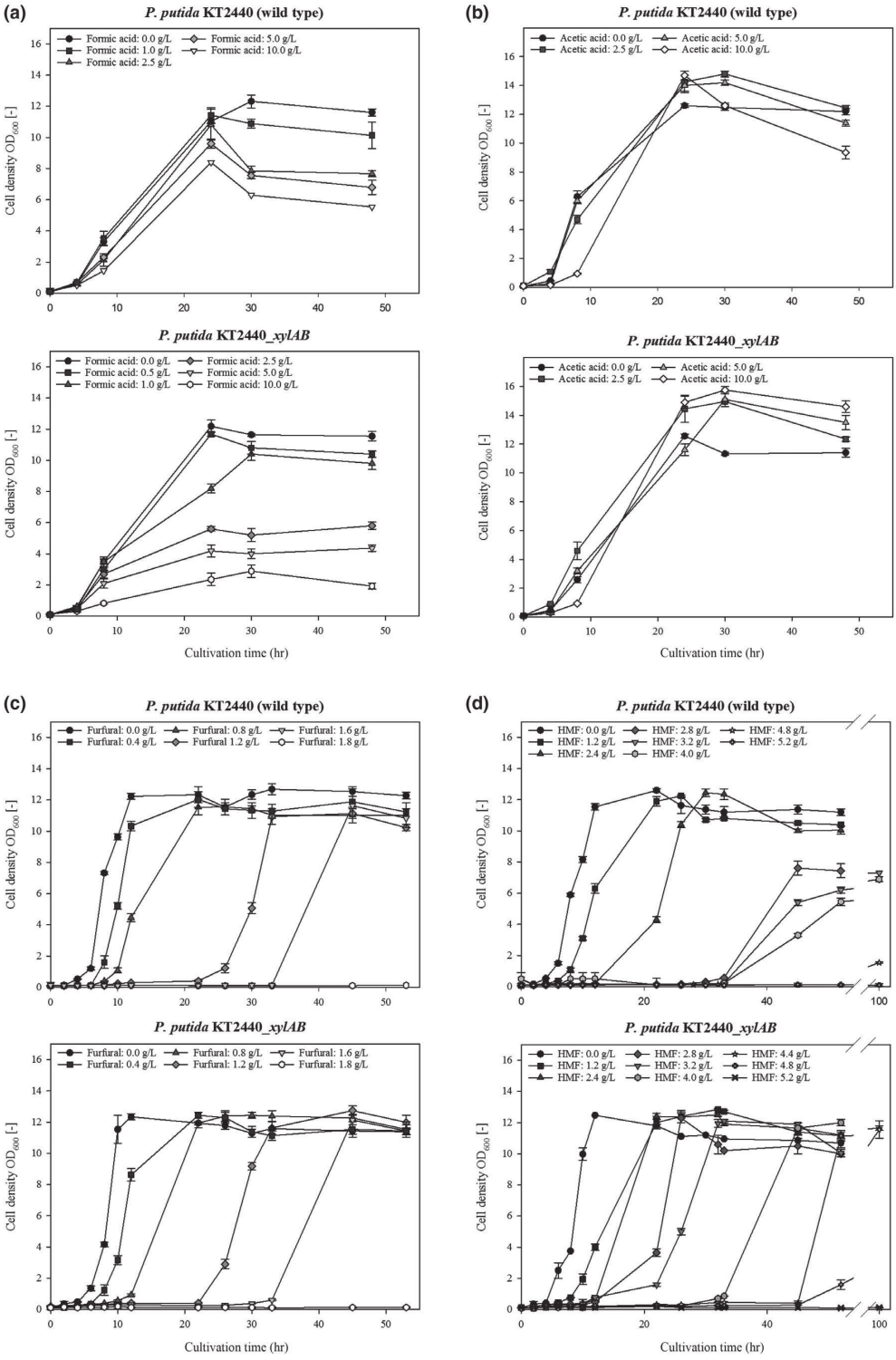

FIGURE 2 Time courses of OD_{600} during cultivation with *Pseudomonas putida* KT2440_*xylAB* and wild type by using glucose as a primary carbon source and adding formic acid (a), acetic acid (b), furfural (c), and hydroxymethylfurfural (HMF; d) to the medium

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

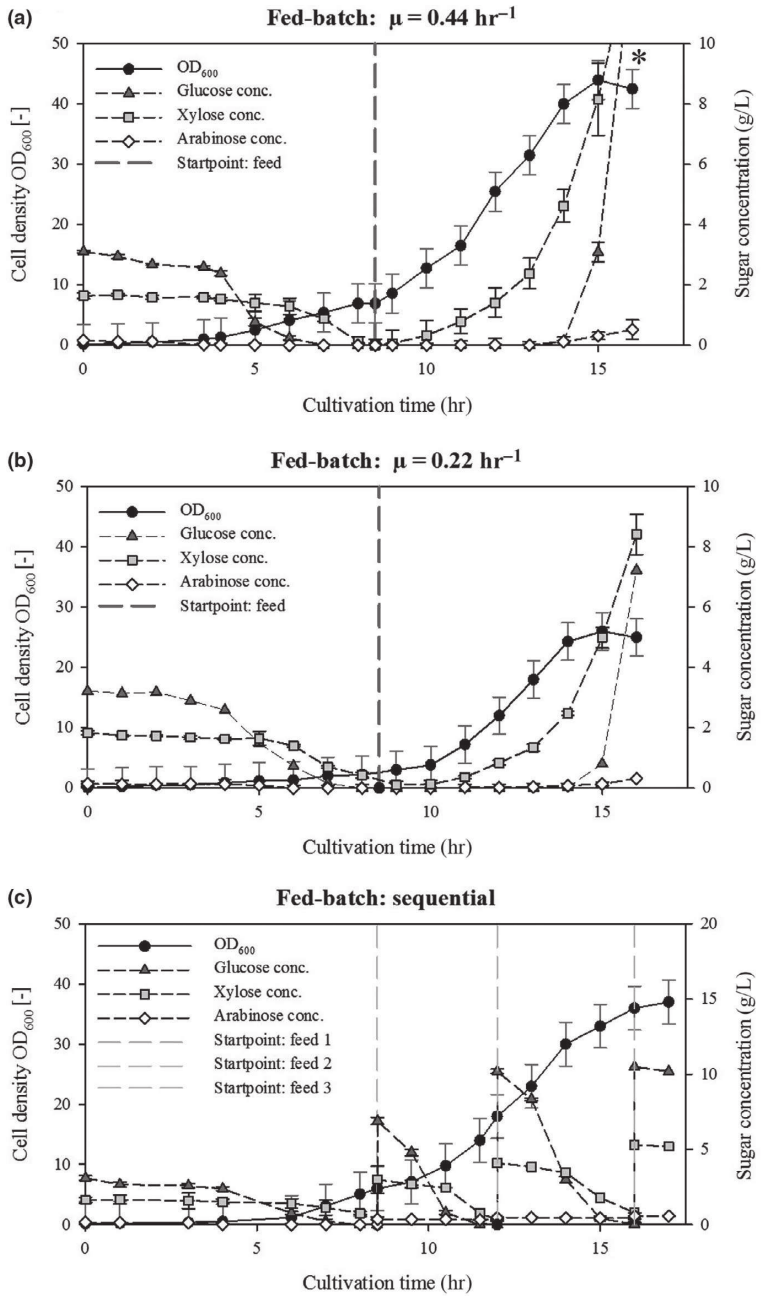

FIGURE 3 Time courses of OD_{600} and sugar concentration during fed-batch bioreactor cultivations with *Pseudomonas putida* KT2440_*xylAB*. (a) Calculated growth rate of $\mu = 0.44$/hr, (b) $\mu = 0.22$/hr, (c) sequential feeding. *Concentration after 16 hr: glucose 23.9 g/L, xylose 13.2 g/L

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.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministry of Science, Research and the Arts of Baden-Württemberg (funding codes 7533-10-5-86A and 7533-10-5-86B) as part of the BBW ForWerts Graduate Program. We thank Matthias Schmidt (Green Sugar AG, Meissen, Germany), Sandra Schläfle, and Ralf Kölling-Paternoga (Institute of Food Science and Biotechnology, Department of Yeast Genetics and Fermentation Technology, University of Hohenheim),

Susanne Zibek and Thomas Hahn (Industrial Biotechnology, Department of Molecular Biotechnology, Fraunhofer Institute for Interfacial Engineering and Biotechnology [IGB], Stuttgart, Germany and Fraunhofer Center for Chemical-Biotechnological Processes [CBP], Leuna, Germany) for the provision of hydrolyzate samples.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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How to cite this article: Horlamus F, Wang Y, Steinbach D, et al. Potential of biotechnological conversion of lignocellulose hydrolyzates by *Pseudomonas putida* KT2440 as a model organism for a bio-based economy. *GCB Bioenergy*. 2019;11:1421–1434. https://doi.org/10.1111/gcbb.12647

2.3 3rd Publication:
One-step bioconversion of hemicellulose polymers to rhamnolipids with *Cellvibrio japonicus*: A proof-of-concept for a potential host strain in future bioeconomy

Horlamus F, Wittgens A, Noll P, Michler J, Müller I, Weggenmann F, Oellig C, Rosenau F, Henkel M, Hausmann R

Global Change Biology Bioenergy 2019, 11:260–268

DOI: 10.1111/gcbb.12542

The purpose of this study was to evaluate *C. japonicus* as a potential host strain for one-step bioconversion of hemicellulose polymers to value-added products. *C. japonicus* could be cultivated on all main lignocellulose monosaccharides as well as xylan polymers as sole carbon source. Exemplary for a value-added product, a one-step conversion of xylan polymers to mono-rhamnolipid biosurfactants with *C. japonicus* was shown after transformation with a plasmid carrying the genes *rhlAB* for rhamnolipid biosynthesis.

Received: 15 May 2018 | Accepted: 4 July 2018

DOI: 10.1111/gcbb.12542

ORIGINAL RESEARCH

WILEY GLOBAL CHANGE BIOLOGY BIOENERGY

One-step bioconversion of hemicellulose polymers to rhamnolipids with *Cellvibrio japonicus*: A proof-of-concept for a potential host strain in future bioeconomy

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Funding information
Fachagentur Nachwachsende Rohstoffe e.V., Grant/Award Number: 22004513

Abstract

The purpose of this study was to evaluate *Cellvibrio japonicus* as a potential host strain for one-step bioconversion of hemicellulose polymers to value-added products. *C. japonicus* could be cultivated on all main lignocellulose monosaccharides as well as xylan polymers as a sole carbon source. This is particularly interesting as most industrially relevant bacteria are neither able to depolymerize wood polymers nor metabolize most hemicellulose monosaccharides. As a result, lignocellulose raw materials typically have to be degraded employing additional processes while the complete conversion of all lignocellulose sugars remains a challenge. Exemplary for a value-added product, a one-step conversion of xylan polymers to mono-rhamnolipid biosurfactants with *C. japonicus* after transformation with the plasmid pSynPro8oT carrying the genes *rhlAB* was demonstrated. As achieved product yields in this one-step bioconversion process are comparably low, many challenges remain to be overcome for application on an industrial scale. Nonetheless, this study provides a first step in the search for establishing a future host strain for bioeconomy, which will ideally be used for bioconversion of lignocellulose polymers with as little exhaustive pretreatment as possible.

KEYWORDS

bioconversion, bioeconomy, *Cellvibrio japonicus*, hemicellulose, lignocellulose, rhamnolipid, xylan

1 | INTRODUCTION

Lignocellulose is a major resource for a bio-based economy as it is the most abundant biological resource of the planet and not a direct competitor to food production. As the structural framework of woody plant cell walls, it consists mainly of the polymers cellulose, hemicellulose, and lignin (Naik, Goud, Rout, & Dalai, 2010). Cellulose consists of glucose monomers linked by β-1,4 glycosidic bonds and has several established applications such as cellulosic fibers for paper or microcrystalline cellulose for food applications (Nsor-Atindana et al., 2017; Walker, 2006) and bioconversion of its depolymerization product glucose is trivial. Lignin, as a complex macromolecule, is the most important renewable source for aromatic polymers and an important

target of research in material science (Upton & Kasko, 2016). Approaches to use microbial conversion of lignin in biotechnological processes will probably not surpass the threshold of feasibility studies in the foreseeable future.

Hemicellulose is the general term for the second polymer in lignocellulose, a group of heteroglycans of several different monomers, such as D–glucose, D–galactose, D–mannose, D-xylose, L-arabinose, as well as sugar acids (Hendriks & Zeeman, 2009). Regarding hemicellulose, xylose is the predominant noncellulosic sugar in hardwoods such as beech, birch or willow and in grasses (Poaceae) such as corn or wheat (Jørgensen, Kristensen, & Felby, 2007). Consequently, xylans are the principal hemicelluloses in these plants (Sjöström, 1993; Willför, Sundberg, Pranovich, & Holmbom, 2005). With a content of 14.9% (total dry weight) in willow (Sassner, Galbe, & Zacchi, 2006) or 18.5% in birch (Hayn, Steiner, Klinger, & Steinmüller, 1993) xylose is an important but mostly underestimated renewable carbon source. It remains mostly unused, as many of the biotechnological important microorganisms do not possess enzymes to break down hemicelluloses such as xylans or to metabolize xylose. Therefore, lignocellulose polymers first have to be degraded by time-consuming and expensive treatments (Hendriks & Zeeman, 2009; Jørgensen et al., 2007; van Dyk & Pletschke, 2012). Due to the reasons listed above, microorganisms which are able to metabolize hemicellulose-related monosaccharides or have the ability to degrade lignocellulose polymers may convey an advantage for efficient bioprocesses. As a huge portfolio of enzymes is necessary, establishing a lignocellulose degrading strain through metabolic engineering is a major challenge. An alternative is the use of organisms which are naturally equipped with a wide range of such enzymes, like the Gram-negative saprophytic soil bacterium *Cellvibrio japonicus* (former name: *Pseudomonas fluorescens subsp. cellulosa*). Many studies in the past showed that *C. japonicus* is able to degrade the main plant cell wall polysaccharides (DeBoy et al., 2008; Gardner, 2016; Gilbert, Jenkins, Sullivan, & Hall, 1987; Hazlewood & Gilbert, 1998; McKie et al., 1997). Previous work demonstrated the genetic accessibility and the possibilities to genetically modify this bacterium (Emami, Nagy, Fontes, Ferreira, & Gilbert, 2002). Expression of recombinant genes in *C. japonicus* was reported via a conjugation based vector system (Gardner & Keating, 2010).

Biosurfactants are microbial surfactants produced using renewable raw materials. One prominent example among them is rhamnolipids. These surface-active glycolipids consist of one or two L-rhamnose linked to one or two hydroxy fatty acids (Bergström, Theorell, & Davide, 1946), and have been used as value-added microbial model products in the past (Müller, Hörmann, Syldatk, & Hausmann, 2010). Rhamnolipids are mostly produced by *Pseudomonas aeruginosa*. As this strain is an opportunistic human pathogen and rhamnolipid production is strongly controlled by complex regulatory systems (Henkel et al., 2013; Pearson, Pesci, & Iglewski, 1997; Rosenau et al., 2010; Wilhelm, Gdynia, Tielen, Rosenau, & Jaeger, 2007), most current research on rhamnolipid production aims at nonpathogenic heterologous production hosts (Cha, Lee, Kim, Kim, & Lee, 2008; Ochsner, Reiser, Fiechter, & Witholt, 1995; Tiso et al., 2016; Wittgens et al., 2011)). In addition, lignocellulose has been proposed as a carbon source for rhamnolipid production in the past (Henkel et al., 2012).

The purpose of this study was to evaluate *C. japonicus* as a potential host strain for one-step bioconversion of lignocellulose polymers and its potential application for the production of value-added products using rhamnolipid biosurfactants as an example.

2 | MATERIALS AND METHODS

2.1 | Chemicals and standards

All chemicals were acquired from Carl Roth GmbH (Karlsruhe, Germany) if not mentioned otherwise. Mono-RL (Rha-C_{10}-C_{10}) standard was obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany) and rhamnolipid standard as mixture from Jeneil Biotech Inc. (Saukville, WI, USA).

2.2 | Media

Cellvibrio japonicus was cultured in minimal salt medium M9 (Harwood & Cutting, 1990) with different carbon sources at concentrations of 0.5% (5 g/L). For cultivations using mannose and arabinose, 0.05% (0.5 g/L) glucose was added to initiate growth. The monosaccharides D-(+)-glucose, D-(+)-galactose, L-(+)-arabinose, D–(+)–mannose and D-(+)–xylose were used. As hemicellulose polymers, corn xylan was used as well as three different beech-xylans obtained from Carl Roth GmbH (Karlsruhe, Germany), SERVA Electrophoresis GmbH (Heidelberg, Germany) and abcr GmbH (Karlsruhe, Germany). For strains containing plasmid, pSynPro8oT_*rhlAB* tetracycline was added as a selection marker at a concentration of 50 μg/ml.

2.3 | Cultivation

For precultures, 25 ml of M9 medium were inoculated with 50 μl of glycerol stock solutions and the main cultures were inoculated with a starting optical density at 600 nm (OD_{600}) of 0.1. The cultivations were performed in 250 ml Erlenmeyer baffled flasks at 30°C and 120 rpm in an incubation shaker (Eppendorf AG, Hamburg, Germany). For storing at –80°C the culture was mixed with glycerol (25% *v/v*), and frozen in liquid nitrogen.

2.4 | Strains and plasmids

Cellvibrio japonicus Ueda107 wild type (formerly classified as *Pseudomonas fluorescens subsp. cellulosa*) was obtained from the National Collection of Industrial Food and Marine Bacteria NCIMB (Aberdeen, UK) listed under strain number 10,462. For expression of genes *rhlAB* required for rhamnolipid biosynthesis plasmid pSynPro8oT carrying a tetracycline resistance was used as described previously (Beuker, Steier, et al., 2016).

2.5 | DNA techniques

Plasmid DNA was isolated using the Midi Plasmid Kit from QIAGEN GmbH (Hilden, Germany). DNA concentration was measured with NanoDrop 2000c spectrophotometer from Thermo Fisher Scientific GmbH (Braunschweig, Germany) at 260 nm. Electrocompetent cells were prepared according to (Troeschel, Drepper, Leggewie, Streit, & Jaeger, 2010). For transformation, 50–100 ng of plasmid DNA and an electroporation device (Eporator, Eppendorf AG, Hamburg, Deutschland) set at 2,400 V was used. Cells were incubated for 3 hr at 30°C and 500 rpm with 500 µl prewarmed SOC medium (Hanahan, 1983). After incubation, 10 and 100 µl culture were plated on agar plates with 50 µg/ml tetracycline antibiotic as selection marker.

2.6 | Analytical methods

2.6.1 | Cell density

Cell growth was determined by measuring optical density at 600 nm (OD_{600}) using a cell density meter (CO8000, Biochrom Limited, Cambridge, United Kingdom) and the culture was diluted with saline (0.9%) as required.

2.6.2 | Rhamnolipid analysis by high-performance thin-layer chromatography (HPTLC)

Sample preparation and extraction for rhamnolipid determination was performed as described previously (Müller et al., 2010). Briefly, rhamnolipids were precipitated from the cell-free extract using 0.01 vol phosphoric acid, extracted twice with ethyl acetate, evaporated to dryness and resolved in acetonitrile. For quantification of rhamnolipids samples were derivatizated with 2,4´-dibromoacetophenone and trimethylamine according to (Cooper & Anders, 1974) as described previously for HPLC (Müller et al., 2010; Schenk, Schuphan, & Schmidt, 1995). Adjustment for the HPTLC was adapted as follows, the derivatization reagent was composed of a 1:1 mixture of 135 mM 2,4´–dibromoacetophenone and 67.5 mM trimethylamine in acetonitrile. Standard and samples were mixed with derivatization reagent in a ratio 1:10 and incubated at 60°C and 2,000 rpm for 90 min in a thermoshaker. For qualitative evaluation with mass spectrometry (MS), samples were not derivatizated. To localize zones of interest derivatizated samples were applied additionally on the HPTLC plate.

Samples were analyzed using an HPTLC system (CAMAG, Muttenz, Switzerland) controlled by the software winCATS, version 1.4.7 (CAMAG). The HPTLC system consisted of an Automatic TLC Sampler 4 (ATS 4), an Automatic Developing Chamber (ADC 2) equipped with a 20 cm × 10 cm twin-trough chamber, a TLC Visualizer and a TLC Scanner 4. HPTLC silica gel 60 plates (Merck, Darmstadt, Germany) were prewashed with methanol, dried in a drying oven (UV 110, Memmert GmbH & Co. KG, Staufen, Germany) at 60°C for 1 hr and stored dust-free until use.

Derivatized samples and standards were applied on 20 x 10 cm plates as 6-mm bands with 8 mm distance from the lower edge and 20 mm distance from the left edge (track distance set to automatic). Application was configured to a filling speed of 15 µl/s and a dosage speed of 150 nl/s. Methanol was used as rinsing solvent. As mobile phase, a mixture of isopropyl acetate/ethanol/water/acetic acid (30:5:2.5:1, *v/v/v/v*) was used. Before development, chamber saturation was adjusted for 10 min, after development, a drying step was performed for 2 min.

For quantitation, the plate was scanned in the absorption mode at UV 263 nm (deuterium lamp) with a scanning speed of 20 mm/s, a resolution of 100 µm per step and a slit dimension of 3 mm x 0.30 mm. Evaluation was performed with the software winCATS applying "polynomial regression mode."

2.6.3 | Mass spectrometry

Zones of interest from the HPTLC plate were directly eluted via the oval elution head (4 x 2 mm) of the TLC–MS Interface (CAMAG) using methanol/water (9:1, *v/v*) including 0.1% formic acid at a flow rate of 0.1 ml/min. The eluent was provided by an Agilent (Waldbronn, Germany) 1,100 HPLC pump and the interface was connected to the MSonline. In the tubing from the interface to the MS, a PEEK inline filter with a 0.5 µm frit was integrated. A G1956B MSD single quadrupole MS with a G1946 atmospheric pressure ionization electrospray (ESI) interface was employed (Agilent). The devices were controlled by the software LC/MSD ChemStation B.04.03 (Agilent). For negative ionization, the parameters used were as follows: capillary voltage 4 kV, skimmer voltage 35 V, lens 2.5 V, quadrupole temperature 100°C, drying gas temperature 300°C, drying gas flow rate 10 L/min and nebulizer gas pressure 40 psig. Total ion chronograms were recorded at *m/z* 100–1,000, using a fragmentor voltage of 100 V, gain

1, threshold 100, and step size 0.1. For evaluation, the spectrum of the plate background at a migration distance comparable to the analyte zone was subtracted from the analyte spectrum.

3 | RESULTS

3.1 | Growth of *Cellvibrio japonicus* on lignocellulose monosaccharides

The wild-type strain of *C. japonicus* was cultured with the main different lignocellulose related monosaccharides. Growth was observed on all these lignocellulose-derived hexoses: glucose, mannose, and galactose. As delineated in Figure 1 the highest growth was reached using glucose with a maximum OD_{600} of 3.2. With mannose, *C. japonicus* showed significantly slower growth. By adding 0.5 g/L glucose as "starter" faster growth was observed at the beginning of the cultivation. This is the result of the metabolization of glucose. Furthermore, growth was detected on galactose with a maximum OD_{600} of 2.2. Furthermore, *C. japonicus* was able to metabolize the two hemicellulose pentoses. When xylose was used as a sole carbon source, similar growth behavior as with glucose was noted with maximum OD_{600} of 3.1. In contrast, no growth was observed when arabinose was used as a carbon source. However, the addition of 0.5 g/L glucose resulted in cell growth with maximum OD_{600} of 2.6. *C. japonicus* was cultivated using softwood xylans as sole sources of carbon. To account for variability of xylans due to origin from different plants as well as different batches, xylans from maize and beech from different sources were compared. Growth behavior with beech-xylans from Carl Roth GmbH & Co. KG and SERVA Electrophoresis GmbH was quite similar compared to growth on xylose reaching a maximum OD_{600} of 2.8 and 2.5, whereas the growth with beech-xylan from abcr GmbH was lower with a maximum OD_{600} of 1.8 and in case of corn xylan from Carl Roth GmbH & Co. KG the lag phase was longer (Figure 1).

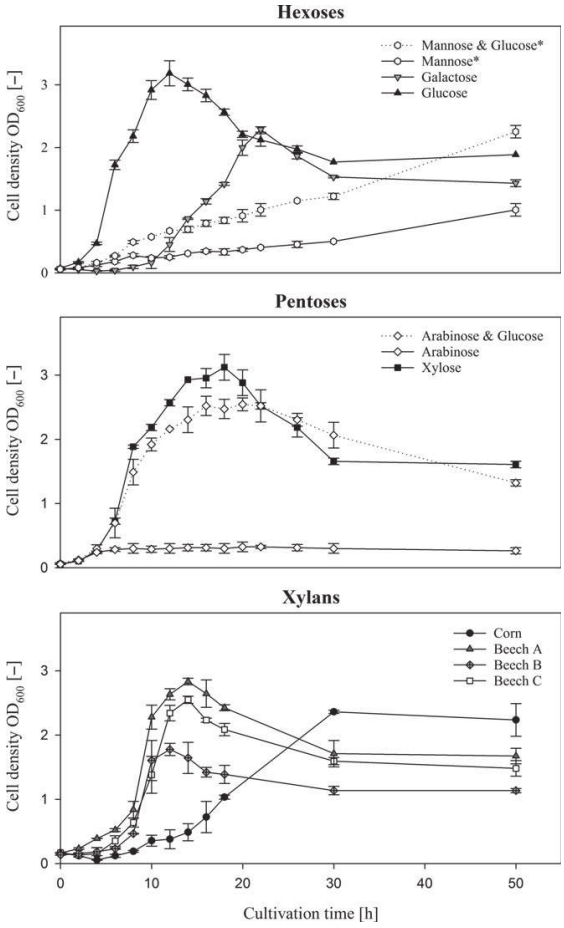

FIGURE 1 Cultivation of *Cellvibrio japonicus* with lignocellulose related hexoses, pentoses and with different xylans (corn from Carl Roth GmbH & Co. KG, beech from Carl Roth GmbH & Co. KG (a), SERVA Electrophoresis GmbH (b) and abcr GmbH (c). Initial sugar concentration was 5 g/L and in case of mannose and arabinose, 0.5 g/L of glucose was added to the respective sugar. * Cultivation was continued until $t = 124$ hr. OD_{600} of mannose as carbon source was 2.0 and for mannose with glucose 2.2

3.2 | Biosynthesis toward rhamnolipid precursor molecules in *C. japonicus*

The formation of rhamnolipid precursors dTDP-L-rhamnose and 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA) by *C. japonicus* was investigated in silico (Table 1) and compared to wild-type rhamnolipid producer *Pseudomonas aeruginosa* PAO1. A full genome sequence of the applied strain *C. japonicus* Ueda107 has been deposited to Database Resources of the National Center for Biotechnology Information (NCBI), and all relevant genes have been annotated in the past (NCBI Resource Coordinators, 2017). However, it should be noted that evidence on protein level is given neither by (NCBI Resource Coordinators, 2017) nor universal protein database (UNIPROT) (The UniProt Consortium, 2017), although both databases list all investigated proteins derived from homology.

Furthermore, the presence of each individual gene from *P. aeruginosa* PAO1 (NCBI:txid1708767) as a natural producer of rhamnolipids, as well as similarity rating was investigated by BLAST analysis in the genome of *C. japonicus* Ueda107 (NCBI:txid498211) (Altschul, Gish, Miller, Myers, & Lipman, 1990). Total sequence similarities above 65% were calculated for all relevant genes

TABLE 1 Biosynthesis toward rhamnolipid precursor molecules in *Cellvibrio japonicus* Ueda107 in comparison with wild-type producer *Pseudomonas aeruginosa* PAO1

	Cellvibrio japonicus			*Pseudomonas aeruginosa* PAO1			
Enzyme	**Annotation (gene)**	**Annotation (protein)**	**Evidence on protein level**	**Gene ID**	**Coverage (%)**	**Identity (%)**	**Score**
dTDP-L-rhamnose biosynthesis							
Glucose−1-phosphate thymidylyltransferase	CJA_RS16570	From homology	No	rmlA	86	67	221
dTDP-D-glucose 4,6-dehydratase	CJA_RS16565	From homology	No	rmlB	85	66	199
dTDP−4-dehydrorhamnose 3,5-epimerase	CJA_RS16580	From homology	No	rmlC	70	65	72
dTDP−4-dehydrorhamnose reductase	CJA_RS16575	From homology	No	rmlD	No significant hit		
3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA) biosynthesis							
3-oxoacyl-(acyl-carrier-protein) reductase	CJA_RS08165	From homology	No	fabG	98	72	639
3-oxoacyl-(acyl-carrier-protein) synthase	CJA_RS00575	From homology	No	fabF	85	67	316
Beta-ketoacyl-ACP synthase	CJA_RS03655	From homology	No	fabB	50[a]	38	147

Notes. Annotated sequences and gene names of *C. japonicus* were extracted from NCBI database**.** Similarity of each individual gene to *Pseudomonas aeruginosa* PAO1 (NCBI:txid1708767) as a natural producer of rhamnolipids was investigated by BLAST alignment analysis.
[a]Truncated fragment, calculated for alignment with highest score.

except for dTDP-4-dehydrorhamnose reductase, for which no significant alignment was possible, and beta-ketoacyl-ACP synthase, for which only a truncated fragment was found in the genome (Table 1).

3.3 | Heterologous production of rhamnolipids with *C. japonicus* from xylan polymers

Cellvibrio japonicus was successfully transformed with the pSynPro8oT plasmid carrying the genes *rhlA* (acyltransferase) and *rhlB* (rhamnosyltransferase I) required for the biosynthesis of mono-rhamnolipids. The resulting recombinant strain *C. japonicus_rhlAB* was cultivated using glucose and xylose in comparison with different xylans as carbon sources (Figure 2). The successful production of mono-rhamnolipids was determined in the culture supernatant by high-performance thin-layer chromatography (HPTLC, Figure 3). The usage of monosaccharides as carbon sources resulted in the production of 4.0 mg/L with glucose and 3.6 mg/L with xylose after 48 hr of cultivation. In comparison, a maximum rhamnolipid concentration of up to 4.9 mg/L could be achieved with xylan from beech as a carbon source.

As expected, production of mono-rhamnolipids could be detected in the transformed strain, but not in the negative control without plasmid (Figure 3). Furthermore, two additional spots could be detected in the extract from the transformed strain: one with a hR_F value of approx. 25 slightly above di-rhamnolipid (Rha-Rha-C_{10}-C_{10}) and another spot

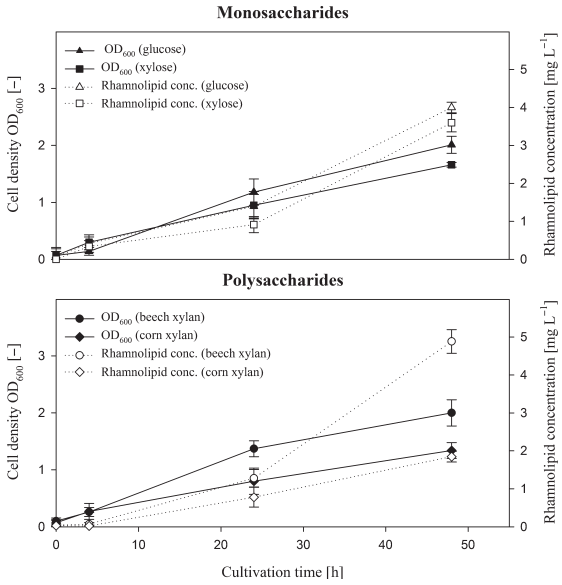

FIGURE 2 Heterologous production of rhamnolipid with *Cellvibrio japonicus* pSynPro8oT_*rhlAB* with lignocellulose monosaccharides and different xylans. Initial concentration of sugars and polymers was 5 g/L

with a hR_F value of approx. 80. No similarities to common rhamnolipid species from *P. aeruginosa* regarding separation behavior could be observed using HPTLC. Furthermore, attempts to identify a rhamnolipid species by

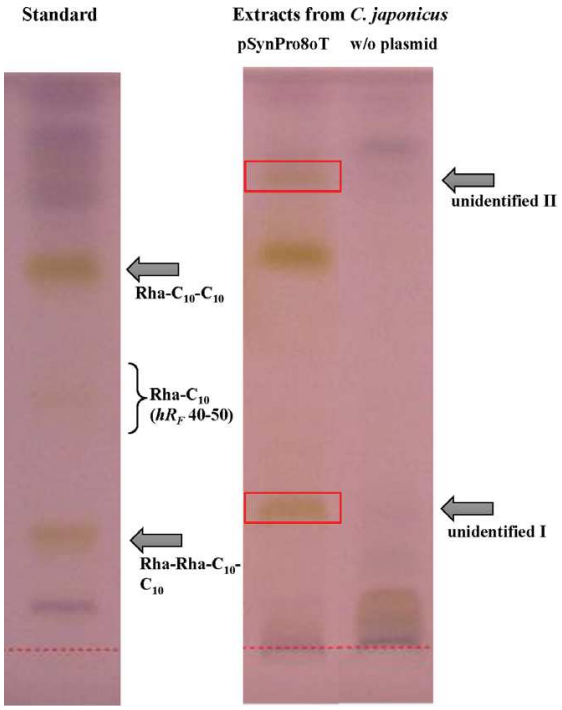

FIGURE 3 HPTLC analysis of rhamnolipid production in *C. japonicus* carrying plasmid pSynPro8oT_*rhlAB*. Rhamnolipid standard (left), extract of culture supernatant grown on xylan as carbon source (middle) and negative control without plasmid (right)

assigning fragment masses in HPTLC-MS spectra did not lead to conclusive results. However, using HPTLC-MS, evidence of formation of Rha-C_{10} was detected at an hR_F value between 40 and 50, which was not detected in wild-type control samples without plasmid (fragment mass of *m/z* 331) (Abdel-Mawgoud, Lépine, & Déziel, 2010; Heyd et al., 2008).

4 | DISCUSSION

4.1 | Hemicellulose as carbon source

Many industrially relevant microorganisms such as *Corynebacterium glutamicum* and *Aspergillus niger* have not a high enzyme portfolio in terms of hemicellulose degradation and metabolization. In this study, it is remarkable that the wild type of *Cellvibrio japonicus* grows on xylose and displays similar growth behavior as with glucose. These results have to be underlined as xylose is the predominant hemicellulose sugar in hardwood and as an example, the total dry weight of willow consists of 14.9% of xylose (Sassner et al., 2006).

Furthermore, the wild type of *C. japonicus* also displays growth on minor hemicellulose components such as galactose. Considering the development of fed-batch processes, which are typically required for high efficient bioprocesses, total consumption of all minor components from crude hemicellulose based substrates is highly favorable. Even though the maximal yield cannot be influenced significantly, this allows to circumvent an accumulation of these components leading to inhibition.

Noteworthy are the results with the hemicellulose polymer xylan as a carbon source. With beech-xylan, similar growth behavior as with glucose or pentoses could be observed. With corn xylan, a longer lag phase could be observed. From the structure, there is a difference between monocotyledons and dicotyledons. Monocotyledons such as corn consist mainly of (glucurono)arabinoxylans, while dicotyledons generally have a significantly lower content of arabinose. It is therefore conceivable that the observed effect of an increased lag phase in case of the culture grown on corn xylan is negatively affected resulting to the more complex enzymatic degradation process required for xylan depolymerization. With regard to these results, *C. japonicus* is a promising potential candidate for development of a one-step bioprocess. Consolidated bioprocesses (CBP) combine the production of enzymes, saccharification, and fermentation in one step. By harnessing the broad portfolio of extracellular hemicellulose-degrading enzymes, a separate process step comprising the preparation and addition of enzymes can be circumvented, as it is common in simultaneous saccharification and fermentation (SSF) processes.

4.2 | Heterologous production of rhamnolipids

Heterologous production of rhamnolipids was reported for different microorganisms with initial concentrations in the range of 10–100 mg/L in the past (Ochsner et al., 1995). Nowadays, a much more efficient production is reported for *Pseudomonas putida* KT2440 with currently achieved concentrations of about 15 g/L (Beuker, Barth, et al., 2016). With the emerging trend of a bio-based economy, novel microorganisms with a broad metabolic spectrum regarding the utilization of lignocellulose polymers are required. As none of the currently employed microorganisms for rhamnolipid production fulfills these requirements, *C. japonicus* may provide an alternative.

Achieved concentrations of rhamnolipids as reported in this study can by far not compete with current heterologous high yield strains and processes. However, considering the history of heterologous rhamnolipid production and achieved concentrations this not only outlines the demand for optimization but also the high optimization potential. As a strategy for optimization, a view on the molecular level could be worthwhile. The transcription could be improved by applying other promoters, in particular by

construction and screening of synthetic promoters optimized for a high efficiency in *C. japonicas* to circumvent native regulatory systems. Another possibility is to improve the translation efficiency by optimization of the Shine-Dalgarno sequence or the codon usage, as several codons for certain amino acids are more commonly used in some organisms than others (Nakamura, Gojobori, & Ikemura, 2000). A further strategy to increase the amounts of rhamnolipids includes the improvement of educt availability, for example by coexpression of responsible genes for dTDP-L-rhamnose biosynthesis, which seems to be a bottleneck in rhamnolipid biosynthesis (Cabrera-Valladares et al., 2006). Furthermore, it should be noted that the reported rhamnolipid concentrations in this study were obtained from shake flask cultivations; therefore, transfer of the process to a bioreactor with controlled feeding could lead to a signification improvement of the process.

Expression of the *rhlAB* operon in *C. japonicus* resulted in the biosynthesis of mono-rhamnolipids. The length of contained 3-hydroxyfatty acids with 10 carbon atoms was expected, as the used *rhl*-genes originating from *P. aeruginosa* possess a specificity mainly for these short-chain lengths independent of the physiological background of the host organism (Wittgens et al., 2018) (Figure 3). Furthermore, analysis of fragments in spectra obtained from HPTLC-MS suggests the production of mono-rhamnolipid congener Rha-C_{10}. This rhamnolipid congener usually represents only a minority in a mixture of rhamnolipids. As the molecular mechanisms of biosynthesis of Rha-C_{10} are not yet fully elucidated (Déziel et al., 1999; Wittgens et al., 2018), the expression system used in this study may be applied as a tool to provide further insight into the mechanisms of biosynthesis for mono-rhamno-mono-lipids. However, it should be noted that obtained signals from HPTLC-MS are comparably weak, yet the fragments could only be detected in extracts from cultures harboring the *rhlAB* expression plasmid but not in the negative control without plasmid (Supporting Information Figure S1).

In this study, the potential of the nonpathogenic bacterium *Cellvibrio japonicus* as a host strain for one-step bioconversion of hemicellulose polymers to value-added products was evaluated. The wild-type strain of *C. japonicus* could be cultivated on all main lignocellulose monosaccharides as well as xylan polymers as sole carbon sources. An example for a value-added product, a one-step conversion of xylan polymers to mono-rhamnolipid biosurfactant with a modified *C. japonicus* was demonstrated. Achieved rhamnolipid concentrations of 4.9 mg/L as reported in this study are still comparably low, which displays that as a future host strain for bioeconomy, many challenges remain to be overcome. Nonetheless, this study outlines the high potential of *C. japonicus* and provides a proof-of-concept for one-step production of rhamnolipids on hemicellulose polymers.

ACKNOWLEDGEMENTS

This work was funded by the Fachagentur Nachwachsende Rohstoffe e.V. [funding code 22004513].

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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: Horlamus F, Wittgens A, Noll P, et al. One-step bioconversion of hemicellulose polymers to rhamnolipids with *Cellvibrio japonicus*: A proof-of-concept for a potential host strain in future bioeconomy. *GCB Bioenergy*. 2019;11:260–268. https://doi.org/10.1111/gcbb.12542

3 Final discussion

The topic of this research project was the biotechnological conversion of lignocellulose hydrolyzates by *Pseudomonas putida* KT2440 and *Cellvibrio japonicus* as model organism for a bio-based economy. Particular emphasis was given to the application of hemicelluloses as a carbon source. Lignocellulose is composed commonly of 20-30% of hemicelluloses, nevertheless the most current bioprocesses are based on glucose derived from cellulose. Two approaches were chosen for this purpose. *P. putida* KT2440 has become a remarkable workhorse for the expression of heterogeneous genes in the last years due to its robustness and versatile metabolism, but it has a poor enzyme profile for metabolizing hemicellulose sugars (Henkel et al. 2012; Loeschcke and Thies 2015; Udaondo et al. 2018). *C. japonicus*, on the other hand, is used as a model organism to elucidate the microbial degradation of lignocellulosic polysaccharides due to its huge portfolio of cellulases and hemicellulases. However it is not applied as biocatalyst in biotechnological processes (Gardner 2016; Gardner and Keating 2012). The results are presented in chapter 2.1 "Growth of engineered *P. putida* KT2440 on glucose, xylose, and arabinose: Hemicellulose hydrolysates and their major sugars as sustainable carbon sources", chapter 2.2 "Potential of biotechnological conversion of lignocellulose hydrolyzates by *P. putida* KT2440 as model organism for a bio-based economy" and chapter 2.3 "One-step bioconversion of hemicellulose polymers to rhamnolipids with *C. japonicus*: A proof-of-concept for a potential host strain in the future bioeconomy.

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

The intent of this publication was to analyze the growth characteristics of *P. putida* KT2440 strains with glucose, xylose and arabinose as single sugars, as mixtures and with lignocellulose hydrolyzates. Furthermore, xylose and arabinose metabolizing strains were developed. In this respect, cultivation experiments were performed in 250-mL Erlenmeyer flasks filled with 25 mL Wilms-KP_i medium. Enzyme kits were applied for sugar analysis.

As expected, *P. putida* KT2440 wild type grew with glucose but not with xylose and arabinose as sole carbon source. Accordingly, *P. putida* KT2440*_xylAB* was engineered. Therefore, the isomerase pathway was chosen, as it was already successfully demonstrated for many bacteria such as *Cornybacterium glutamicum* (Kawaguchi et al. 2006; Meiswinkel et al. 2013), *Zymomonas monobilis* (Zhang et al. 1995) and *P. putida* (Meijnen et al. 2008). The Weimberg pathway is another way to metabolize xylose via D-xylose to α-ketoglutarate. This was already demonstrated for *P. putida* S12 (Meijnen et al. 2009) but was not included in this publication. Furthermore, the arabinose metabolizing strain *P. putida* KT2440*_araBAD* was constructed. So far, no other publication has reported that this metabolic pathway has been tested for *P. putida*. Both recombinant strains grew on xylose, as well as, on arabinose. *P. putida* KT2440*_xylAB* performed slightly better on xylose, whereas the strain with the araBAD operon performed slightly better on arabinose. Meijnen and coworkers have previously reported that the strain *P. putida* S12*_xylAB* grew on arabinose, but in the case of *P. putida* KT2440*_araBAD* no publication could be found (Meijnen et al. 2008). Accordingly, there must be putative enzymes for the conversion of L-ribulose to D-xylulose, but the exact metabolic route is still unknown. All in all, two different xylose and arabinose metabolizing *P. putida* KT2440 strains were successfully engineered. Future work should focus on the construction of strains for degrading hemicelluloses, like xylans or mannans, as well as, on hemicellulose-related disaccharides like xylobiose.

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

After the successfull engineering of xylose and arabinose- metabolizing *P. putida* KT2440 strains, the focus of this publication was to evaluate the potential of this strain for the biotechnological conversion of lignocellulose hydrolyzates. *P. putida* KT2440*_xylAB* was chosen as it grew slightly better on xylose and on wheat straw hydrolyzates than the strain with the *araBAD* operon. First, *P. putida* KT2440*_xylAB* was cultivated on hydrolyzates of different origins and the bacterial growth behavior was analyzed. Second, the content of major inhibitors in lignocellulose hydrolyzates was detected and their inhibitory effect on bacterial growth was described. Third, a fed-batch cultivation strategy in a bioreactor with lignocellulose hydrolyzates as carbon source was displayed.

Depending on the applied biomass and process, lignocellulose hydrolyzates typically exhibit a high variation in sugar composition and in the inhibitors formed during hydrolysis. This displays a major obstacle to its efficient use as a carbon source for biotechnological processes. With this background hydrolyzates of different plant species, chemical and enzymatic hydrolysis and different pretreatments (organosolv, steam explosion) were tested. It was demonstrated that *P. putida* KT2440*_xylAB* reached similar growth rates with several lignocellulose hydrolyzates as with glucose. A major advantage of this strain was its high compatibility with acetic acid under neutral conditions, since many hydrolyzates contain this organic acid. No inhibition growth was observed up to the highest tested concentration of 10 g/L. Compared to acetic acid, this strain was more sensitive to formic acid and the growth rate was weaker from a concentration of 1.0 g/L. In lignocellulose hydrolyzates the formic acid concentration is usually below 1.0 g/L (Chandel et al. 2011; Palmqvist and Hahn-Hägerdal 2000a, 2000b) and it could become only problematic with highly efficient fed-batch bioprocesses with highly concentrated sugar solutions. Furfural aldehydes turned out to be critical. The bacterial growth was negatively influenced starting from a furfural concentration of 0.4 g/L and starting from a HMF concentration of 1.2 g/L. This was not reflected in a reduction in growth rates but in an extension of the lag phase. One reason for this could be the reduction of furfural aldehydes to less toxic alcohols, which is practiced by many bacteria (Guarnieri et al. 2017). Organic acid and furanaldehydes are mostly

considered as inhibitors in biotechnological processes (Palmqvist and Hahn-Hägerdal 2000a, 2000b), although they could also serve as potential carbon sources. For this reason, the purpose of subsequent work should not solely be to increase the stability of *P. putida* KT2440 strains against these compounds, but also to supplement metabolic pathways to enable the strains to utilize them as carbon sources. *P. putida* KT2440 were able to metabolize acetic acid. Guarnieri and coworkers closed the catabolism pathway of HMF and furfural in *P. putida* KT2440 by genomic integration of the *hmf* genes cluster. These strains were more stable against these two furfural aldehydes and could grow to a certain extent on furfural and HMF (Guarnieri et al. 2017).

In the next step, fed-batch processes in a 2-L-bioreactor were performed with wheat straw hydrolzyates treated by a steam explosion process followed by enzymatic hydrolyzation as carbon source. The hypothesis that during cultivation with sugar mixtures *P. putida* KT2440*_xylAB* only metabolizes the pentose sugars once glucose has been consumed could be confirmed. This issue prevents on the one hand the complete consumption of the sugars and on the other hand accumulation of sugars can have an inhibitory effect on the microbial growth. As a counterstrategy, a step-by-step fed-batch process was explored. The feed was only added when xylose was almost consumed. With this process strategy, a complete metabolism of the pentose sugars xylose and arabinose was attained. In addition, no relevant lag phases were observed and growth during pentose sugar metabolism was detected. A maximum OD_{600} of 35 was measured after 15 hr. One other answer to this issue is to engineer strains for simultaneous consumption of sugars. Recently Dvořák and de Lorenzo used the xylose H^+ symporter encoded of *xylE* from *E. coli* additionally to *xylAB* genes for engineering *P. putida* EM42. In addition, the *gcd* gene was deleted to prevent oxidation of xylose to the corresponding sugar acid xylonate in periplasm through glucose dehydrogenase. This strain was able to co-utilize glucose and xylose (Dvořák and de Lorenzo 2018). In this context, it would be interesting to know whether the simultaneous metabolism of several sugars in the bacterium causes stress symptoms especially at high feed rates. According to an ^{13}C-based metabolic flux ratio analysis of Nikel and coworkers, only 10% of glucose passed the inner membrane directly via the ABC transporter and 90% of glucose is first phosphorylated by the Gcd in the periplasm before it passes the inner cell membrane (Nikel et al. 2015). Using only the ABC transporter, caused by deleting the *glc* gene, could be a bottleneck especially in case of high feed rates. A third

conceivable strategy could be a consortium consisting of glucose metabolizing strains (wild type) and glucose negative pentose metabolizing strains (engineered strains). Del Castillo and coworkers isolated *P. putida* KT2440 mutants which were unable to use glucose as sole carbon source. These strains had insertions in e*dd* (encodes phosphogluconate dehydratase) and *eda* (encodes KDPG aldolase) (Del Castillo et al. 2007). However, future projects could focus on the further development and comparison of these three strategies for establishing efficient simultaneous sugar metabolism processes.

In summary, these are according to our knowledge the first studies in which *P. putida* KT2440 was cultivated on lignocellulose/hemicellulose hydrolyzates. Several of the selected hydrolyzates turned out as suitable carbon source for *P. putida* KT2440. Furfural and HMF concentration proved to be critical factors for the selection of lignocellulose hydrolysates. Furthermore, a feeding strategy to prevent the diauxic-like growth pattern is delineated.

3.3 One-step bioconversion of hemicellulose polymers to rhamnolipids with *Cellvibrio japonicus*: A proof-of-concept for a potential host strain in future bioeconomy

While the first two publications were concerned with the application of hemicellulose monosaccharides as carbon sources, this publication dealt with the direct employment of hemicellulose polymers as carbon sources. This approach allows the costly pretreatment and hydrolysis to be reduced. In addition, the problem of inhibitors resulting from these processes is avoided. As the degradation of hemicellulose polymers requires a large portfolio of enzymes, strain engineering is a challenge in this case. Microorganisms that are naturally able to degrade and metabolize hemicellulosic polymers, such as the soil bacteria *C. japonicus*, could be alterative approaches. Cultivations were performed in shaking flasks filled with minimal salt medium and with lignocellulose monosaccharides and xylan as carbon sources. The wild type strain was able to grow on glucose, galactose, mannose, xylose and xylans as the sole carbon source. With arabinose, bacterial growth could only be detected with the addition of small amounts of glucose as a "starting jumper". The growth performance of *C. japonicus* with xylose and xylans was similar to that of glucose and with

corn xylan there was a longer lag phase than with beech xylan. In this context it should be mentioned that the structure of xylans varies between monocotyledons, such as corn, and dicotyledons, such as beech. (Glucorono)arabinoxylans are the dominant xylans in monocotyledons while hemicelluloses of dicetyldons generally have a significant lower content of arabinose and consist mainly of glucoronoxylans and xyloglucans. Accordingly, variations in requirements for the degradation of the different xylans or the increased arabinose content may be a reason for the longer lag phase in cultivations with corn hydrolyzates. Considering the results of the cultivation experiments, *C. japonicus* could be a promising microbial biocatalyst for consolidated bioprocesses (CBP), which combines enzyme production, saccharification and fermentation in one step (Hasunuma and Kondo 2012; Salehi Jouzani and Taherzadeh 2015). The next step was to develop product producing strains and therefore rhamnolipids were chosen. According to silico analysis with the NCBI database, *C. japonicus* has all relevant genes to metabolize the precursors dTDP-L-rhamnose and 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA). Therefore, mono-rhamnolipid producing strain was engineered with *rhlA* (encodes acetyltransferase) and *rhlB* (encodes rhamnosyltransferase I). *C. japonicus_rhlAB* expressed with glucose, xylose or xylan as carbon source similar amounts of rhamnolipids. Surprisingly, the maximum rhamnolipid concentration with xylan (4.9 mg/L) was higher than with glucose (4.0 g/L), which underlines that the hemicellulose polymer xylan is an excellent carbon source for *C. japonicus*. Nevertheless, these results are not yet satisfactory with respect to the current rhamnolipid concentrations of 15 g/L achieved with *P. putida* KT2440 and glucose as carbon source (Beuker et al. 2016a). On the one hand, improvements at the molecular level are conceivable. Transcription could be enhanced by using other promoters, in particular screening and construction of synthetic promoters to bypass bottlenecks in native regulatory systems in *C. japonicus*. In addition, dTDP-L-rhamnose biosynthesis is a frequently reported bottleneck and therefore a heterologous expression of responsible genes might be helpful (Cabrera-Valladares et al. 2006). On the other hand, cultivations were carried out in Erlenmeyer flasks, meaning that optimizations of the process in bioreactor under controlled conditions has considerable potential for improvement. Another possibility for further research projects could be the use of cellulases and hemicellulases from *C. japonicus* for the development of lignocellulose degrading *P. putida* KT2440 strains. In this context, an emphasis should be put on the transport system, because it could be a potentially bottleneck and very little literature is available. *P. putida* KT2440 proved to be a suitable biocatalyst

for rhamnolipids and recombinant strains with *araBAD*- and *xylAB* operon were able to metabolize hemicellulose monosaccharides. Consequently, engineering of *P. putida* strains capable of metabolizing hemicelluloses or related disaccharides should therefore be the next step on the ladder to create efficient hosts for the lignocellulosic biorefinery.

Overall, the recombinant strain *C. japonicus_rhlAB* was successfully engineered for expression of rhamnolipids. To the best of our knowledge, this is the first time that rhamnolipids have been produced using xylans as carbon source. As the achieved rhamnolipid concentration of 4.9 g/L is comparatively low the results could be regarded as a proof-of-concept for the one-step bioconversion of xylans to rhamnolipids as an example for value-added products.

3.4 Conclusion

P. putida KT2440 strains were successfully engineered by applying the *xylAB*- and the *araBAD* operon for metabolizing hemicellulose monosaccharides. These constructed recombinant strains were able to grow on several lignocellulose hydrolyzates with similar growth rates as with glucose. The inhibitory effect of main toxic compounds of lignocellulose hydrolyzates on bacterial growth was characterized and furfural and HMF were identified as critical key factors. A fed-batch bioreactor cultivation with wheat straw hydrolyzates as carbon source was carried out and, in this context, a diauxic-like growth pattern was revealed. In order to avoid the accumulation of pentose sugars a step-by-step feeding strategy was presented. In summary, these are the first shown bioprocesses with *P. putida* KT2440 as host strain and hemicellulose hydrolyzates as carbon source. On this basis, models for the ecological and economic evaluation of these processes could be developed in subsequent projects. In addition, the design of polymer metabolizing *P. putida* KT2440 strains should now be considered.

The growth pattern of *C. japonicus* on lignocellulose related monosaccharides as well as on xylans was analyzed and *C. japonicus* was transformed with the *rhlAB* genes to express mono-rhamnolipid biosurfactants. To our knowledge it is the first published one-step bioconversion of xylan to rhamnolipids. Since the achieved product yields were comparatively low, there are still obstacles to overcome in order to establish an economically profitable process. All in all, these studies demonstrated the potential of *P. putida* KT2440

and *C. japonicus* as potential host strains for the lignocellulosic biorefinery in a frame of Bioeconomy.

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Annex 3

Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic

Biotechnological conversion of lignocellulose hydrolyzates - model microorganisms for a bio-based economy

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……...

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Place, Date

Signature

Annexes

Annex 1: Abbrevations list of enzymes and transporters of Figure 3

PEP: phosphoenolpyruvate, KDPG: 2-keto-3-deoxy-6-phosphogluconate, glucose/mannose ABC transporter: GtsABC/MalK, GntP: gluconate H^+symporter, KguT: 2 ketogluconate H^+symporter, Gcd: glucose dehydrogenase, Gnl: gluconolactonase, Gad: D-gluconate/ D-galactonate dehydratase, GnuK: gluconokinase, KguK: putative 2-ketogluconokinase, Zwf: glucose-6-phosphate-1-dehydrogenase, PgI: Glucose-6-phosphate isomerase, GntZ: 6-phosphogluconate dehydrogenase, KguD: 2-ketogluconate 6-phosphate reductase, Edd: phosphogluconate dehydratase, Eda: 2-dehydro-3-deoxy-phosphogluconate (KDPG) aldolase, GlK: glucokinase, GnuK: Gluconokinase, KguK: putative 2-ketogluconokinase, KguD: 2-ketogluconate 6-phosphate reductase, Pgi: glucose-6-phosphate isomerase, Fbp: fructose-1,6-bisphosphatase, Fba: fructose-1,6-bisphosphate aldolase, TbiA: triose phosphate isomerase, Tal: transaldolase, Gap: glyceraldehyde-3-phosphate dehydrogenase, Pgk: phosphoglycerate kinase, Gpm: phosphoglycerate mutase, Eno: enolase, PykF: pyruvate kinase, PpsA: phosphoenolpyruvate synthetase, AceEF: pyruvate dehydrogenase E1 and E2 component, Ppc: phosphoenolpyruvate carboxylase, Pck: phosphoenolpyruvate carboxykinase, GltA: citrate synthase, AcnA: aconitate hydratase, Icd: isocitrate dehydrogenase (NADP), SucAB: 2-oxoglutarate dehydrogenase E1 and E2 component, SucCD: succinyl-CoA synthetase, Sdh: succinate dehydrogenase, Fum: fumarate hydratase, Mdh: malate dehydrogenase, AceA: isocitrate lyase, AceB/GlcB: malate synthase, AraE, H^+ symporter, AraA: L-isomerase, AraB: L-ribulokinase, AraD: L-ribulokinase-5-phosphate 4-epimerase, XylE, H^+ symporter, XylA: xylose isomerase, XylB: xylulokinase, YagF: D-xylonate dehydratase, XylX: 2-keto deoxy D-xylonate dehydratase, XylA: α KGSA dehydrogenase, YagE: 2-dehydro-3-deoxy-D-pentonate

aldolase, AldA: aldehyde dehydrogenase, ManI: mannose 6 phosphate isomerase, HK: Hexokinase, AldA: mannose 6 phosphate isomerase/mannose 1 phosphate guanylyltransferase, GalP: H^+ symporter, GalM: aldose 1-epimerase, GalK:galactokinase, GalT: Galactose-1-phosphate uridylyltransferase, GalE: UDP-glucose-4-epimerase, PgM: phosphoglucomutase