

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

Felix Horlamus¹ | Andreas Wittgens^{2,3}  | Philipp Noll¹ | Jan Michler¹ | Inga Müller¹ |
Fabiola Weggenmann¹ | Claudia Oellig⁴ | Frank Rosenau^{2,3,5} | Marius Henkel¹  |
Rudolf Hausmann¹

¹Department of Bioprocess Engineering (150k), Institute of Food Science and Biotechnology, University of Hohenheim, Stuttgart, Germany

²Institute for Pharmaceutical Biotechnology, Ulm University, Ulm, Germany

³Ulm Center for Peptide Pharmaceuticals (U-PEP), Ulm University, Ulm, Germany

⁴Institute of Food Chemistry, University of Hohenheim, Stuttgart, Germany

⁵Department Synthesis of Macromolecules, Max-Planck-Institute for Polymer Research, Mainz, Germany

Correspondence

Marius Henkel, Department of Bioprocess Engineering (150k), Institute of Food Science and Biotechnology, University of Hohenheim, Fruwirthstraße 12, 70599 Stuttgart, Germany.
Email: Marius.Henkel@uni-hohenheim.de

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 pSynPro80T 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-C₁₀-C₁₀) 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, pSynPro80T_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 (OD₆₀₀) 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 μ l prewarmed SOC medium (Hanahan, 1983). After incubation, 10 and 100 μ l culture were plated on agar plates with 50 μ g/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 derivatized 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 derivatized. To localize zones of interest derivatized 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 \times 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 \times 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 μ l/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 μ m per step and a slit dimension of 3 mm \times 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 \times 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 μ m 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 chromatograms 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 OD₆₀₀ 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 OD₆₀₀ 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 OD₆₀₀ 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 OD₆₀₀ 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 OD₆₀₀ of 2.8 and 2.5, whereas the growth with beech-xylan from abcr GmbH was lower with a maximum OD₆₀₀ of 1.8 and in case of corn xylan from Carl Roth GmbH & Co. KG the lag phase was longer (Figure 1).

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).

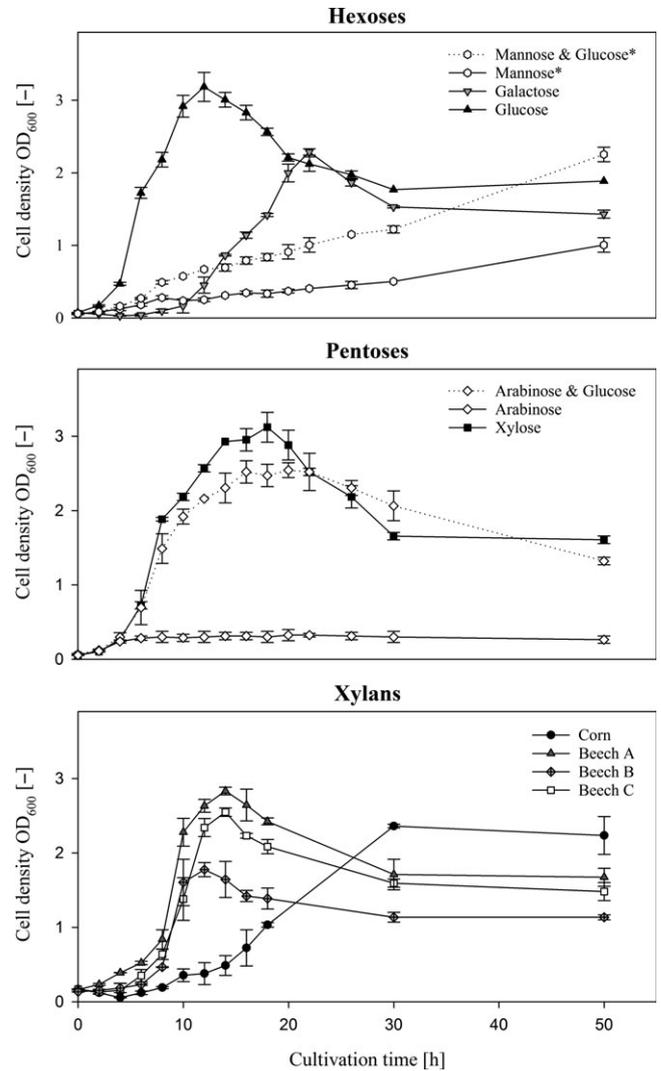


FIGURE 1 Cultivation of *Cellvibrio japonicus* with lignocellulose related hexoses, pentoses and with different xylans (corn from Carl Roth GmbH & Co. KG, beech from Carl Roth GmbH & Co. KG (a), SERVA Electrophoresis GmbH (b) and abcr GmbH (c)). Initial sugar concentration was 5 g/L and in case of mannose and arabinose, 0.5 g/L of glucose was added to the respective sugar. * Cultivation was continued until $t = 124$ hr. OD₆₀₀ of mannose as carbon source was 2.0 and for mannose with glucose 2.2

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

Enzyme	<i>Cellvibrio japonicus</i>			<i>Pseudomonas aeruginosa</i> PAO1			
	Annotation (gene)	Annotation (protein)	Evidence on protein level	Gene ID	Coverage (%)	Identity (%)	Score
dTDP-L-rhamnose biosynthesis							
Glucose-1-phosphate thymidyltransferase	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.

^aTruncated 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-C₁₀-C₁₀) and another spot

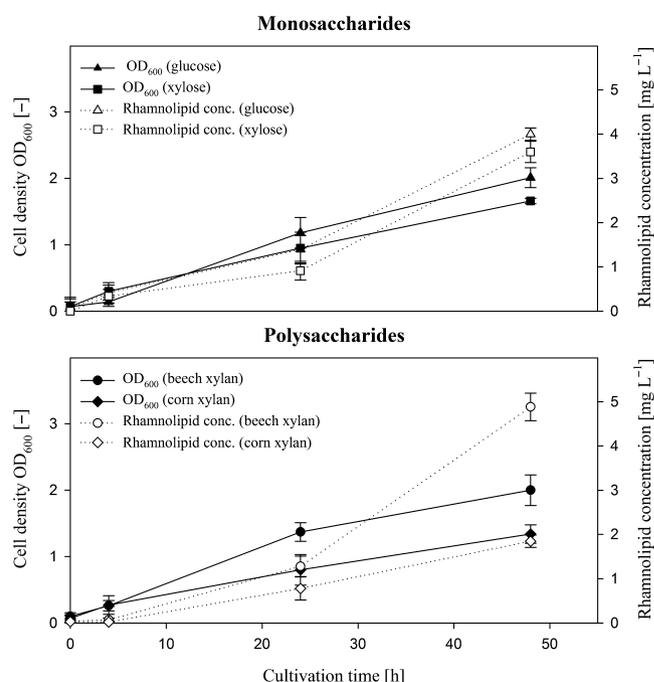


FIGURE 2 Heterologous production of rhamnolipid with *Cellvibrio japonicus* pSynPro8oT_rhlAB with lignocellulose monosaccharides and different xylans. Initial concentration of sugars and polymers was 5 g/L

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

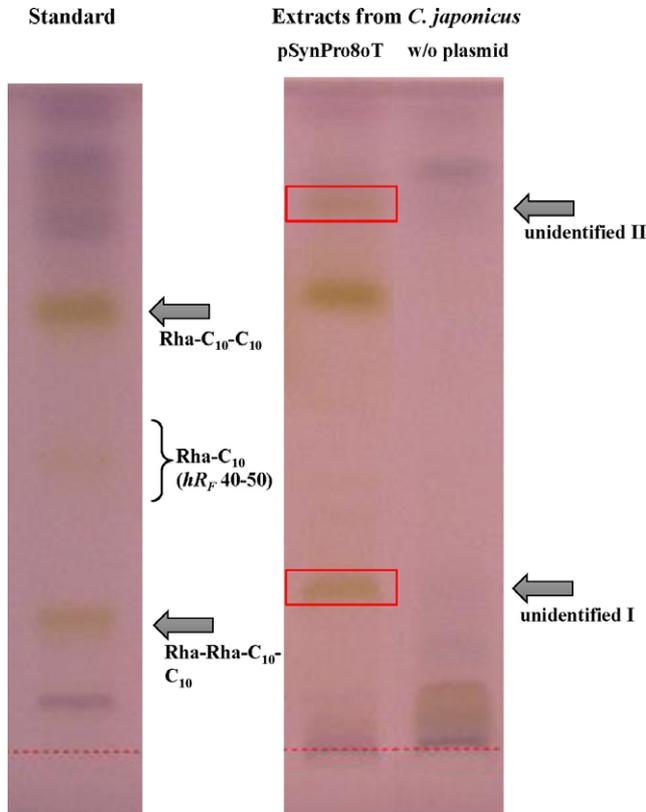


FIGURE 3 HPTLC analysis of rhamnolipid production in *C. japonicus* carrying plasmid pSynPro8oT_rhlAB. Rhamnolipid standard (left), extract of culture supernatant grown on xylan as carbon source (middle) and negative control without plasmid (right)

assigning fragment masses in HPTLC-MS spectra did not lead to conclusive results. However, using HPTLC-MS, evidence of formation of Rha-C₁₀ 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 significant 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-C₁₀. This rhamnolipid congener usually represents only a minority in a mixture of rhamnolipids. As the molecular mechanisms of biosynthesis of Rha-C₁₀ 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].

ORCID

Andreas Wittgens  <http://orcid.org/0000-0001-6771-4380>

Marius Henkel  <http://orcid.org/0000-0002-5343-9661>

REFERENCES

- Abdel-Mawgoud, A. M., Lépine, F., & Déziel, E. (2010). Rhamnolipids. Diversity of structures, microbial origins and roles. *Applied Microbiology and Biotechnology*, 86, 1323–1336. <https://doi.org/10.1007/s00253-010-2498-2>
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215, 403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2)
- Bergström, S., Theorell, H., & Davide, H. (1946). On a metabolic product of *Pseudomonas pyocyanea*, pyolipic acid, active against *Mycobacterium tuberculosis*. *Arkiv för Kemi, Mineralogi och Geologi*, 23A, 1–12.
- Beuker, J., Barth, T., Steier, A., Wittgens, A., Rosenau, F., Henkel, M., & Hausmann, R. (2016). High titer heterologous rhamnolipid production. *AMB Express*, 6, 124.
- Beuker, J., Steier, A., Wittgens, A., Rosenau, F., Henkel, M., & Hausmann, R. (2016). Integrated foam fractionation for heterologous rhamnolipid production with recombinant *Pseudomonas putida* in a bioreactor. *AMB Express*, 6, 11.
- Cabrera-Valladares, N., Richardson, A. P., Olvera, C., Treviño, L. G., Déziel, E., Lépine, F., & Soberón-Chávez, G. (2006). Monorhamnolipids and 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs) production using *Escherichia coli* as a heterologous host. *Applied Microbiology and Biotechnology*, 73, 187–194. <https://doi.org/10.1007/s00253-006-0468-5>
- Cha, M., Lee, N., Kim, M., & Lee, S. (2008). Heterologous production of *Pseudomonas aeruginosa* EMS1 biosurfactant in *Pseudomonas putida*. *Bioresource Technology*, 99, 2192–2199. <https://doi.org/10.1016/j.biortech.2007.05.035>
- Cooper, M. J., & Anders, M. W. (1974). Determination of long chain fatty acids as 2-naphthacyl esters by high pressure liquid chromatography and mass spectrometry. *Analytical Chemistry*, 46, 1849–1852. <https://doi.org/10.1021/ac60348a035>
- DeBoy, R. T., Mongodin, E. F., Fouts, D. E., Tailford, L. E., Khouri, H., Emerson, J. B., ... Nelson, K. E. (2008). Insights into plant cell wall degradation from the genome sequence of the soil bacterium *Cellvibrio japonicus*. *Journal of Bacteriology*, 190, 5455–5463. <https://doi.org/10.1128/JB.01701-07>
- Déziel, E., Lépine, F., Dennie, D., Boismenu, D., Mamer, O. A., & Villemur, R. (1999). Liquid chromatography/mass spectrometry analysis of mixtures of rhamnolipids produced by *Pseudomonas aeruginosa* strain 57RP grown on mannitol or naphthalene. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1440, 244–252. [https://doi.org/10.1016/S1388-1981\(99\)00129-8](https://doi.org/10.1016/S1388-1981(99)00129-8)
- Emami, K., Nagy, T., Fontes, C. M. G. A., Ferreira, L. M. A., & Gilbert, H. J. (2002). Evidence for temporal regulation of the two

- Pseudomonas cellulosa* xylanases belonging to glycoside hydrolase family 11. *Journal of Bacteriology*, *184*, 4124–4133. <https://doi.org/10.1128/JB.184.15.4124-4133.2002>
- Gardner, J. G. (2016). Polysaccharide degradation systems of the saprophytic bacterium *Cellvibrio japonicus*. *World Journal of Microbiology and Biotechnology*, *32*, 121. <https://doi.org/10.1007/s11274-016-2068-6>
- Gardner, J. G., & Keating, D. H. (2010). Requirement of the type II secretion system for utilization of cellulosic substrates by *Cellvibrio japonicus*. *Applied and Environmental Microbiology*, *76*, 5079–5087. <https://doi.org/10.1128/AEM.00454-10>
- Gilbert, H. J., Jenkins, G., Sullivan, D. A., & Hall, J. (1987). Evidence for multiple carboxymethylcellulase genes in *Pseudomonas fluorescens* subsp. *cellulosa*. *Molecular and General Genetics*, *210*, 551–556. <https://doi.org/10.1007/BF00327211>
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology*, *166*, 557–580. [https://doi.org/10.1016/S0022-2836\(83\)80284-8](https://doi.org/10.1016/S0022-2836(83)80284-8)
- Harwood, C. R., & Cutting, S. M. (1990). *Chemically defined growth media and supplements* (p. 548). Chichester, UK: Wiley.
- Hayn, M., Steiner, W., Klinger, R., & Steinmüller, H. (Eds.) (1993). Basic research and pilot studies on the enzymatic conversion of lignocellulosics.
- Hazlewood, G. P., & Gilbert, H. J. (1998). Structure and function analysis of *Pseudomonas* plant cell wall hydrolases. *Progress in Nucleic Acid Research and Molecular Biology*, *61*, 211–241.
- Hendriks, A. T. W. M., & Zeeman, G. (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology*, *100*, 10–18. <https://doi.org/10.1016/j.biortech.2008.05.027>
- Henkel, M., Müller, M. M., Kügler, J. H., Lovaglio, R. B., Contiero, J., Syldatk, C., & Hausmann, R. (2012). Rhamnolipids as biosurfactants from renewable resources: Concepts for next-generation rhamnolipid production. *Process Biochemistry*, *47*, 1207–1219. <https://doi.org/10.1016/j.procbio.2012.04.018>
- Henkel, M., Schmidberger, A., Kühnert, C., Beuker, J., Bernard, T., Schwartz, T., ... Hausmann, R. (2013). Kinetic modeling of the time course of N-butyl-L-homoserine lactone concentration during batch cultivations of *Pseudomonas aeruginosa* PAO1. *Applied Microbiology and Biotechnology*, *97*, 7607–7616. <https://doi.org/10.1007/s00253-013-5024-5>
- Heyd, M., Kohnert, A., Tan, T.-H., Nusser, M., Kirschhöfer, F., Brenner-Weiss, G., ... Berensmeier, S. (2008). Development and trends of biosurfactant analysis and purification using rhamnolipids as an example. *Analytical and Bioanalytical Chemistry*, *391*, 1579–1590. <https://doi.org/10.1007/s00216-007-1828-4>
- Jørgensen, H., Kristensen, J. B., & Felby, C. (2007). Enzymatic conversion of lignocellulose into fermentable sugars. Challenges and opportunities. *Biofuels, Bioproducts and Biorefining*, *1*, 119–134. <https://doi.org/10.1002/bbb.4>
- McKie, V. A., Black, G. W., Millward-Sadler, S. J., Hazlewood, G. P., Laurie, J. I., & Gilbert, H. J. (1997). Arabinanase A from *Pseudomonas fluorescens* subsp. *cellulosa* exhibits both an endo- and an exo- mode of action. *Biochemical Journal*, *323* (Pt. 2), 547–555.
- Müller, M. M., Hörmann, B., Syldatk, C., & Hausmann, R. (2010). *Pseudomonas aeruginosa* PAO1 as a model for rhamnolipid production in bioreactor systems. *Applied Microbiology and Biotechnology*, *87*, 167–174. <https://doi.org/10.1007/s00253-010-2513-7>
- Naik, S. N., Goud, V. V., Rout, P. K., & Dalai, A. K. (2010). Production of first and second generation biofuels. A comprehensive review. *Renewable and Sustainable Energy Reviews*, *14*, 578–597. <https://doi.org/10.1016/j.rser.2009.10.003>
- Nakamura, Y., Gojobori, T., & Ikemura, T. (2000). Codon usage tabulated from international DNA sequence databases. Status for the year 2000. *Nucleic Acids Research*, *28*, 292. <https://doi.org/10.1093/nar/28.1.292>
- NCBI Resource Coordinators (2017). Database resources of the national center for biotechnology information. *Nucleic Acids Research*, *45*, D12.
- Nsor-Atindana, J., Chen, M., Goff, H. D., Zhong, F., Sharif, H. R., & Li, Y. (2017). Functionality and nutritional aspects of microcrystalline cellulose in food. *Carbohydrate Polymers*, *172*, 159–174. <https://doi.org/10.1016/j.carbpol.2017.04.021>
- Ochsner, U. A., Reiser, J., Fiechter, A., & Witholt, B. (1995). Production of *Pseudomonas aeruginosa* rhamnolipid biosurfactants in heterologous hosts. *Applied Environmental Microbiology*, *61*, 3503–3506.
- Pearson, J. P., Pesci, E. C., & Iglewski, B. H. (1997). Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *Journal of Bacteriology*, *179*, 5756–5767. <https://doi.org/10.1128/jb.179.18.5756-5767.1997>
- Rosenau, F., Isenhardt, S., Gdynia, A., Tielker, D., Schmidt, E., Tielken, P., ... Jaeger, K. E. (2010). Lipase LipC affects motility, biofilm formation and rhamnolipid production in *Pseudomonas aeruginosa*. *FEMS Microbiology Letters*, *309*, 25–34. <https://doi.org/10.1111/j.1574-6968.2010.02017.x>
- Sassner, P., Galbe, M., & Zacchi, G. (2006). Bioethanol production based on simultaneous saccharification and fermentation of steam-pretreated *Salix* at high dry-matter content. *Enzyme and Microbial Technology*, *39*, 756–762. <https://doi.org/10.1016/j.enzmictec.2005.12.010>
- Schenk, T., Schuphan, I., & Schmidt, B. (1995). High-performance liquid chromatographic determination of the rhamnolipids produced by *Pseudomonas aeruginosa*. *Journal of Chromatography A*, *693*, 7–13. [https://doi.org/10.1016/0021-9673\(94\)01127-Z](https://doi.org/10.1016/0021-9673(94)01127-Z)
- Sjöström, E. (1993). *Wood chemistry. Fundamentals and applications*. London: Academic Press.
- The UniProt Consortium (2017). UniProt: The universal protein knowledgebase. *Nucleic Acids Research*, *45*, D158–D169.
- Tiso, T., Sabelhaus, P., Behrens, B., Wittgens, A., Rosenau, F., Hayen, H., & Blank, L. M. (2016). Creating metabolic demand as an engineering strategy in *Pseudomonas putida*–Rhamnolipid synthesis as an example. *Metabolic Engineering Communications*, *3*, 234–244. <https://doi.org/10.1016/j.meten.2016.08.002>
- Troeschel, S. C., Drepper, T., Leggewie, C., Streit, W. R., & Jaeger, K.-E. (2010). Novel tools for the functional expression of metagenomic DNA. *Methods in Molecular Biology (Clifton, N.J.)*, *668*, 117–139.
- Upton, B. M., & Kasko, A. M. (2016). Strategies for the conversion of lignin to high-value polymeric materials. *Review and Perspective. Chemical Reviews*, *116*, 2275–2306. <https://doi.org/10.1021/acs.chemrev.5b00345>
- van Dyk, J. S., & Pletschke, B. I. (2012). A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes–factors affecting enzymes, conversion and

- synergy. *Biotechnology Advances*, 30, 1458–1480. <https://doi.org/10.1016/j.biotechadv.2012.03.002>
- Walker, J. C. F. (2006). *Primary wood processing. Principles and practice* (p. 596). Dordrecht: Springer.
- Wilhelm, S., Gdynia, A., Tielen, P., Rosenau, F., & Jaeger, K. E. (2007). The autotransporter esterase EstA of *Pseudomonas aeruginosa* is required for rhamnolipid production, cell motility, and biofilm formation. *Journal of Bacteriology*, 189, 6695–6703. <https://doi.org/10.1128/JB.00023-07>
- Willför, S., Sundberg, A., Pranovich, A., & Holmbom, B. (2005). Polysaccharides in some industrially important hardwood species. *Wood Science and Technology*, 39, 601–617. <https://doi.org/10.1007/s00226-005-0039-4>
- Wittgens, A., Santiago-Schuebel, B., Henkel, M., Tiso, T., Blank, L. M., Hausmann, R., ... Rosenau, F. (2018). Heterologous production of long-chain rhamnolipids from *Burkholderia glumae* in *Pseudomonas putida*-a step forward to tailor-made rhamnolipids. *Applied Microbiology Biotechnology*, 102, 1229–1239. <https://doi.org/10.1007/s00253-017-8702-x>
- Wittgens, A., Tiso, T., Arndt, T. T., Wenk, P., Hemmerich, J., Müller, C., ... Blank, L. M. (2011). Growth independent rhamnolipid

production from glucose using the non-pathogenic *Pseudomonas putida* KT2440. *Microbial Cell Factories*, 10, 80. <https://doi.org/10.1186/1475-2859-10-80>

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>