Metabolic engineering of *Pseudomonas putida* for production of the natural sweetener 5-ketofructose from fructose or sucrose by periplasmic oxidation with a heterologous fructose dehydrogenase

Karen Wohlers, Astrid Wirtz, Alexander Reiter, Marco Oldiges, Meike Baumgart and Michael Bott

1IBG-1: Biotechnology, Institute of Bio- and Geosciences, Forschungszentrum Jülich, Jülich, 52425, Germany.
2Institute of Biotechnology, RWTH Aachen University, Aachen, 52062, Germany.
3The Bioeconomy Science Center (BioSC), Forschungszentrum Jülich, Jülich, D-52425, Germany.

Summary

5-Ketofructose (5-KF) is a promising low-calorie natural sweetener with the potential to reduce health problems caused by excessive sugar consumption. It is formed by periplasmic oxidation of fructose by fructose dehydrogenase (Fdh) of *Gluconobacter japonicus*, a membrane-bound three-subunit enzyme containing FAD and three haemes as prosthetic groups. This study aimed at establishing *Pseudomonas putida* KT2440 as a new cell factory for 5-KF production, as this host offers a number of advantages compared with the established host *Gluconobacter oxydans*. Genomic expression of the *fdhSCL* genes from *G. japonicus* enabled synthesis of functional Fdh in *P. putida* and successful oxidation of fructose to 5-KF. In a batch fermentation, 129 g l⁻¹ 5-KF were formed from 150 g l⁻¹ fructose within 23 h, corresponding to a space-time yield of 5.6 g l⁻¹ h⁻¹. Besides fructose, also sucrose could be used as substrate for 5-KF production by plasmid-based expression of the invertase gene *inv1417* from *G. japonicus*. In a bioreactor cultivation with pulsed sucrose feeding, 144 g 5-KF were produced from 358 g sucrose within 48 h. These results demonstrate that *P. putida* is an attractive host for 5-KF production.

Introduction

Consumption of food and beverages with added sugars like sucrose or high-fructose corn syrup is linked to health problems like obesity, diabetes type 2 and cardiovascular diseases (Rippe and Angelopoulos, 2016; Caracho et al., 2017). Reduction of added sugar consumption can be supported with non-nutritive sweeteners (Acero et al., 2020). As such, 5-ketofructose (5-KF) is an interesting product. It is a naturally occurring compound that has a similar taste and sweetness as fructose (Herweg et al., 2018), but is not metabolized by the human body (Wyrobnik and Wyrobnik, 2006) and, as recently shown, not metabolized by 15 prominent bacterial species of the human gut microbiome (Schiessl et al., 2021). 5-KF therefore meets crucial demands for a new sweetener. 5-KF can be synthesized biologically via fructose oxidation catalyzed by the fructose dehydrogenase (Fdh) of *Gluconobacter japonicus* (Ameyama et al., 1981). This membrane-bound dehydrogenase is a heterotrimeric enzyme consisting of a small subunit FdhS with a Tat signal peptide, a large subunit FdhL with a covalently bound flavin adenine dinucleotide (FAD), and a cytochrome c subunit FdhC with three haem-binding motifs CXCH, a Sec-signal peptide and a C-terminal transmembrane helix that anchors the entire periplasmic Fdh complex in the cytoplasmic membrane. FdhL lacks a signal peptide and is probably secreted into the periplasm pickback with FdhS via the Tat export system (Kawai et al., 2013). The industrially used cell factory *Gluconobacter oxydans* contains no endogenous Fdh activity (Ameyama et al., 1981) and the genome does not contain homologs of *fdhSCL* (Prust et al., 2005; Kranz et al., 2017). However, plasmid-based heterologous expression of the *fdhSCL* genes from *G. japonicus* in *G. oxydans* led to much higher Fdh activities than observed for wild-type *G. japonicus* (Kawai et al., 2013). Consequently, the subsequent studies on 5-KF production used recombinant strains of *G. oxydans*.

Equipped with various membrane-bound dehydrogenases (Deppenmeier et al., 2002; Peters et al., 2013), *G. oxydans* is an established host in industrial biotechnology for the periplasmic oxidation of substrates in a chemo-, stereo- and regio-specific manner (Reichstein et al., 2021).
and Grüssner, 1934; De Muynck et al., 2007). In previous studies, G. oxydans with plasmid-encoded Fdh reached high 5-KF production rates and in a fed-batch process, 5-KF titres of almost 500 g l⁻¹ with a yield of up to 0.98 g₅-KF/gfructose were obtained (Herweg et al., 2018; Siemen et al., 2018). Hence, G. oxydans is a suitable host for 5-KF production from fructose. However, G. oxydans also has a number of limitations. It has a very low growth yield compared with other bacteria (Kiefer et al., 2017) and culture media have to be supplemented with yeast extract or other complex nutrient sources in order to obtain fast growth. The number of genetic tools is also limited, although an efficient deletion system (Kostner et al., 2013) and plasmids for constitutive and inducible expression (Kallnık et al., 2010; Fricke et al., 2020) are available. Our knowledge on many aspects of metabolism and in particular regulation in G. oxydans is very limited compared with other bacteria used as multipurpose production hosts in biotechnology, although there is continuous progress (Bringer and Bott, 2016; Schweikert et al., 2021). These features impede the development of G. oxydans production strains by metabolic engineering and prompted us to search for an alternative host for 5-KF production that lacks the limitations described above.

In this study, we analysed the capabilities of Pseudomonas putida KT2440 to serve as production strain for 5-KF. P. putida is a metabolically versatile and robust organism for which plenty of knowledge and many tools and techniques for genetic engineering and heterologous expression are available (Loeschcke and Thies, 2015; Zobel et al., 2015; Martínez-García and de Lorenzo, 2017; Nikel and de Lorenzo, 2018). P. putida shares a number of features with G. oxydans that make it a promising host for 5-KF production. Like G. oxydans, P. putida KT2440 is a strictly aerobic proteobacterium, which contains two membrane-bound dehydrogenases oxidizing glucose to gluconate and 2-ketogluconate in the periplasm. The glucose dehydrogenase (Gcd) is a PQO-dependent enzyme (An and Moe, 2016), whereas gluconate dehydrogenase (Gad) consists of a cytochrome c subunit with a Sec-signal peptide, an FAD-containing subunit without a signal peptide, and a third small subunit with a Tat-signal peptide (Kumar et al., 2013; Winsor et al., 2016). These properties resemble those of Fdh of G. japonicus and suggest that functional expression of the fdhSCL genes in P. putida might be possible.

In contrast to G. oxydans, the oxidized products formed via periplasmic glucose oxidation by P. putida do not accumulate in the medium but the majority is taken up and metabolized in the cytoplasm. Similar to G. oxydans, P. putida lacks a complete Embden-Meyerhof-Parnas pathway and uses the EDEMP cycle for sugar metabolism (del Castillo et al., 2007; Nikel et al., 2015), whereas G. oxydans employs a partially cyclic pentose phosphate pathway as major route and the Entner-Doudoroff pathway is dispensable (Hanke et al., 2013; Richhardt et al., 2013). P. putida, in contrast to G. oxydans, possesses a complete tricarboxylic acid cycle, allowing complete oxidation of acetyl-CoA to CO₂. Differences also exist with respect to the respiratory chain. P. putida possesses a proton-pumping complex I-type NADH dehydrogenase, two non-proton-pumping type II NADH dehydrogenases, two ubiquinol oxidases (cytochrome bo₃ and a cyanide-insensitive bd-type oxidase CIO), a cytochrome bc complex and three cytochrome c oxidases (cytochrome aa₃ and two cytochrome cbb₃ oxidases). G. oxydans possesses only a non-proton-pumping NADH dehydrogenase and two quinol oxidases. Consequently, the flexibility of the respiratory chain and the capabilities for proton-motive force generation are much lower for G. oxydans than for P. putida. As a consequence of these metabolic differences, the biomass yield of P. putida (about 0.5 g/g glucose) is about 5-fold higher than the one of G. oxydans (about 0.1 g/g glucose) and also the growth rates reported for P. putida (about 0.5–0.7 h⁻¹) surpass that of G. oxydans (about 0.3–0.5 h⁻¹) (del Castillo et al., 2007; Ebert et al., 2011; Richhardt et al., 2012; Nikel et al., 2015; Kiefer et al., 2017). Another important difference to G. oxydans is that P. putida grows well in minimal media and does not require complex medium components.

P. putida can utilize fructose as carbon source. Fructose is taken up as fructose 1-phosphate via the PEP-dependent phosphotransferase system PTSFrU composed of the two fusion proteins FruA (EII-B-EIIIC) and FruB (EI-HP-EIIIA). Fructose 1-phosphate enters central carbon metabolism after phosphorylation by the kinase FruK to fructose 1,6-bisphosphate (Chavarría et al., 2013). Besides fructose, sucrose is an interesting cheap substrate for 5-KF production, which is present for example in high amounts in sugar beet molasses (Sjölin et al., 2019). Sucrose is naturally not metabolized by P. putida KT2440, but strains were constructed which were able to utilize sucrose by expression of the cscA and cscB genes of Escherichia coli W, encoding an invertase and a sucrase permease, respectively (Löwe et al., 2017), or by expression of the Pseudomonas protegens Pf-5 cscRABY gene cluster for sucrose uptake and metabolism (Löwe et al., 2020).

The aim of this study is the evaluation of P. putida as a new host for 5-KF production. As first step, we integrated the fdhSCL genes of G. japonicus into the chromosome of P. putida KT2440 via Tn7 integration. The recombinant P. putida::fdhSCL was able to efficiently oxidize fructose to 5-KF. In order to utilize sucrose as substrate for 5-KF production, the G. japonicus inv1417
gene encoding a periplasmic invertase (Hoffmann et al., 2020) was expressed in P. putida::fdhSCL and the resulting strain was able to grow on sucrose as sole carbon source and produce 5-KF from the disaccharide. In summary, we generated a potent 5-KF production strain of P. putida and thus showed that this organism is a suitable host for products requiring periplasmic oxidation.

Results and discussion

Generation of P. putida::fdhSCL and test for 5-KF production

To assess whether heterologous expression of the G. japonicus fructose dehydrogenase genes enables P. putida to oxidize fructose to 5-KF, the fdhSCL cluster was integrated into the genome of P. putida KT2440. For stable and strong expression, we used a pBG14g-derived vector for site-directed Tn7 integration of fdhSCL under control of the strong constitutive synthetic 14g promoter followed by a BCD2 linker that serves as a translational coupler (Zobel et al., 2015). BCD stands for bicistronic design. The BCD2 DNA sequence includes a ribosome binding site preceding a small ORF of 17 codons, which also includes the ribosome binding for the target gene to be expressed, in our case fdhS. The stop codon of this small ORF includes the A of the ATG start codon of the target gene. Translational couplers were shown to reduce effects of the target gene on translation (Mutalik et al., 2013).

The recombinant strain P. putida::fdhSCL and its parental wild type were cultivated in mineral salts medium (MSM) with 100 mM fructose either as sole carbon source or in combination with 20 mM glucose to test for 5-KF production (Fig. 1). Glucose was added to enable faster growth and potentially increase yield and rate of 5-KF production. The wild type consumed only a small portion of the fructose (35 ± 4 mM) when grown either on fructose alone or with 20 mM glucose as additional carbon source (22 ± 4 mM) and did not form 5-KF. P. putida::fdhSCL consumed the entire fructose within 48 h of cultivation and formed 57 ± 4 mM 5-KF when cultivated with fructose alone and 67 ± 4 mM 5-KF when cultivated with fructose and glucose. An example HPLC chromatogram demonstrating 5-KF production in MSM with glucose and fructose as carbon sources is shown in Fig. S1. The molar yields (5-KF formed/fructose consumed) were 0.59 ± 0.02 in medium with only fructose and 0.68 ± 0.03 in glucose-supplemented medium. Although the molar yield related to the sum of fructose and glucose consumed (0.56 ± 0.03) was slightly lower compared with medium with fructose alone, glucose enabled much faster 5-KF production due to a reduced lag phase and an increased growth rate. Hence, glucose addition was also used in the following experiments.

In all cultures an acidification was observed. P. putida oxidizes a large fraction of the consumed glucose initially to gluconate, a fraction of which can remain in the medium and contribute to the acidification (Nikel et al., 2015; Kohlstedt and Wittmann, 2019). A preliminary screening for organic acids in culture supernatants via dilute-and-shoot mass spectrometry (Reiter et al., 2021) modified for detection of organic acids qualitatively identified gluconate, pyruvate and several intermediates of the TCA cycle (citrate, isocitrate, succinate, malate), suggesting that the acidification is due to the excretion of a mixture of organic acids. The pH decrease is important for 5-KF production, since Fdh has a pH optimum of about 4 (Kawai et al., 2013).

The results described above show that P. putida is a suitable alternative host for 5-KF production and is able to functionally express the fdhSCL genes of G. japonicus. This is not self-evident as it requires covalent haem attachment to Sec-secreted FdhC, covalent FAD attachment to FdhL and Tat-dependent secretion of the FdhS-FdhL complex. In order to quantify Fdh activity, enzyme assays were performed with cell-free extracts using a spectrophotometric assay (Ameyama and Adachi, 1982) in which ferricyanide serves as electron acceptor and is reduced to ferrocyanide. Ferrocyanide is subsequently quantified as Prussian blue as described in the Experimental procedures section. For P. putida::fdhSCL, a specific Fdh activity of 1.23 ± 0.08 µmol min⁻¹ (mg protein)⁻¹ was determined, which is comparable to the specific Fdh activity measured for G. oxydans IK003.1-igr3::fdhSCL (1.24 ± 0.15 µmol min⁻¹ (mg protein)⁻¹) that served as positive control. No Fdh activity was detected for the parent strains of P. putida and G. oxydans that do not harbour the fdhSCL genes.

Bioreactor cultivation of P. putida::fdhSCL with 150 g l⁻¹ fructose

The next experiment aimed at determining the potential of P. putida::fdhSCL for 5-KF production when cultivated under controlled conditions in a bioreactor with a high fructose concentration of 150 g l⁻¹ (833 mM). For the cultivation in a DASGIP bioreactor, 1 l MSM medium pH 7 containing 150 g l⁻¹ fructose and 3.6 g l⁻¹ glucose was used. After the initial acidification phase, the pH was kept at pH 5.0 by automated addition of KOH. To prevent foam formation, headspace gassing was applied at a flow rate of 1 vvm and initial stirring at 500 rpm. The dissolved oxygen concentration (DO) was controlled at 30%. The medium was inoculated using overnight shake flask precultures in MSM with 150 g l⁻¹ fructose and 3.6 g l⁻¹ glucose. Four biological replicates were performed (Fig. S2) and a representative result of one of these cultivations is shown in Fig. 2. Despite the high
Sugar concentration, a growth rate of 0.48 ± 0.02 h⁻¹ was observed. Glucose was completely consumed within the first 8 h of cultivation. The pH decreased from 7 to 5 within about 11 h (Fig. 2). 5-KF production correlated with fructose consumption. Within the first 4 h of cultivation, no 5-KF was produced, which might be due to the acidic pH optimum of Fdh (Kawai et al., 2013). After 23 h, fructose had been completely consumed and 129 ± 5 g l⁻¹ 5-KF (mean value and standard deviation of four biological replicates) had been formed, resulting in a yield of 0.88 ± 0.01 g 5-KF/g fructose and a space time yield of 5.60 ± 0.22 g l⁻¹ h⁻¹. In our previous study with the G. oxydans strain IK003.1-igr3::fdhSCL, which also contains a genomically encoded Fdh, a 5-KF yield of 0.84 g/g was achieved within 27 h (Battling et al., 2020). Consequently, P. putida::fdhSCL shows a comparable performance for 5-KF production as G. oxydans IK003.1-igr3::fdhSCL, but only requires a minimal medium and not a medium with yeast extract as G. oxydans. A comparison of relevant parameters of the two production strains is shown in Table 1.

**Biotransformation with resting cells of P. putida and G. oxydans**

To further compare P. putida and G. oxydans regarding their ability to produce 5-KF, resting cells of P. putida::fdhSCL and G. oxydans IK003.1-igr3::fdhSCL were used for the biotransformation of fructose to 5-KF. For this purpose, cell suspensions with an OD₆₀₀ = 3 prepared in 100 mM potassium phosphate buffer pH 6 containing 150 g l⁻¹ fructose were incubated at 30°C and 180 r.p.m. Both strains showed very similar conversion rates of 1.81 ± 0.1 g l⁻¹ h⁻¹ for G. oxydans IK003.1-igr3::fdhSCL and 1.80 ± 0.03 g l⁻¹ h⁻¹ for P. putida::fdhSCL (Fig. 3). No activity loss was observed during the 48 h
Growth, pH, sugar consumption and 5-KF formation of *P. putida::fdhSCL* in a batch cultivation. The strain was cultivated in 1 l MSM with 150 g l\(^{-1}\) fructose and 3.6 g l\(^{-1}\) glucose in a DASGIP bioreactor at 30°C and DO ≥ 30%. After the initial acidification phase, the pH was kept at pH 5 by addition of KOH. For inoculation, overnight shake precultures in MSM with 150 g l\(^{-1}\) fructose and 3.6 g l\(^{-1}\) glucose were used. Fructose, glucose, and 5-KF concentrations in the culture supernatants were determined by HPLC.

A representative example of four biological replicates (Fig. S2) is shown here.

**Table 1.** Comparison of bioreactor cultivations of *G. oxydans* IK003.1-igr3::fdhSCL and *P. putida::fdhSCL*

| Parameter                      | G. oxydans IK003.1-igr3::fdhSCL | P. putida::fdhSCL |
|--------------------------------|---------------------------------|-------------------|
| Final OD\(_{600}\)            | n.d.                            | 0.48 ± 0.02       |
| Yield (g 5-KF g\(^{-1}\) fructose) | 0.84                            | 0.88 ± 0.01       |
| Space time yield (g l\(^{-1}\) h\(^{-1}\)) | 4.37                            | 5.60 ± 0.22       |

**Expansions of the substrate spectrum for 5-KF production to sucrose**

Sucrose represents a cheaper substrate for 5-KF production than fructose and we therefore aimed to construct a *P. putida::fdhSCL* derivative with the ability to utilize sucrose. *P. putida* wild type is unable to metabolize and grow on sucrose, but strains engineered for growth on sucrose were reported (Löwe *et al.*, 2017; Lowe *et al.*, 2020). However, these studies used cytosolic invertases, that is CscA from *E. coli* and CscA from *P. protegens*, whereas we aimed for a periplasmic invertase, which provides fructose for Fdh without the necessity for fructose export from the cytoplasm. Hence, we selected an invertase recently identified in *G. japonicus* LMG1417, which has a high Km for sucrose (63 ± 11 mM), but the highest specific activity (2300 U mg\(^{-1}\)) of all mesophilic invertases. After heterologous inv1417 expression in *G. oxydans*, 40% of the activity was found in the periplasm and 60% in the cytoplasm (Hoffmann *et al.*, 2020). According to *in silico* prediction, Inv1417 contains a Tat signal peptide (Hoffmann *et al.*, 2020). However, as the protein is not known to contain a cofactor, secretion via the Sec machinery cannot be excluded. The *G. japonicus* inv1417 gene was cloned into the expression plasmid pBT-T under the control of the constitutive *P_\text{tac*} promoter. *P. putida::fdhSCL* transformed with pBT-T-inv1417 was cultivated in MSM with 100 mM sucrose as sole carbon source to test whether the invertase is active in *P. putida* and whether efficient 5-KF formation from sucrose can be achieved with this enzyme (Fig. 4).

*P. putida::fdhSCL* (pBT-T-inv1417) grew on sucrose as sole carbon source, but initial growth was slower compared with cultivations with glucose and fructose (see Fig. 1D). Since Inv1417 has an acidic pH optimum at around pH 5 (Hoffmann *et al.*, 2020) and invertase activity is crucial for growth on sucrose as sole carbon source, we compared cultures with an initial pH of 7.0 (Fig. 4A) with cultures having an initial pH of 6.5 (Fig. 4B). The lower initial pH resulted in faster sucrose cleavage, faster 5-KF production and a lower final cell density. After 46.5 h of cultivation, the cultures with a start pH of 6.5 reached an OD\(_{600}\) of 4.7 ± 0.1 compared with 6.6 ± 0.3 for the cultures with a start pH of 7.0. Besides faster 5-KF production, the reduced start pH also led to an increased molar yield (5-KF/sucrose) of 0.93 ± 0.02 compared with 0.82 ± 0.01 obtained for the cultures with a start pH of 7.0.

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As shown in Fig. 4A and B, both fructose and glucose were formed in the first 11 h of the cultivation with roughly similar kinetics. However, whereas the fructose was completely consumed again by conversion to 5-KF, glucose remained in the medium, reaching final concentrations of 54.3 ± 0.4 mM in the cultures with an initial pH of 7 and 65.1 ± 0.3 mM in the cultures with an initial pH of 6.5. This suggests that glucose consumption is inhibited by acidic pH. We could support this assumption by further growth experiments in which we either doubled the buffer capacity of the medium or adjusted the pH after 24 h to 6.5 with NaOH. In both cases, the higher pH led to a higher glucose consumption and increased OD<sub>600</sub> values (Fig. S3). The major pathway for glucose catabolism in <i>P. putida</i> is the periplasmic oxidation to gluconate, which is then taken up and metabolized in the cytoplasm (Nikel et al., 2015; Kohlstedt and Wittmann, 2019). Hence, the membrane-bound glucose dehydrogenase Gcd plays a crucial role in glucose catabolism. For Gcd of <i>P. putida</i>, the pH optimum has not been determined to our knowledge. However, in recent studies on lactobionic acid production with <i>Pseudomonas tae</i>trolens it was reported that the PQQ-dependent glucose dehydrogenase is responsible for the oxidation of lactose to lactobionic acid (Oh et al., 2020). In a subsequent study of the same group, it was shown that lactobionic acid production with <i>P. tae</i>trolens worked best at pH values above 6, suggesting that the optimum pH of the PQQ-dependent glucose dehydrogenase is in this range (Kim et al., 2020). The PQQ-dependent glucose dehydrogenase of <i>P. tae</i>trolens (GenBank: KMM82267.1) shows 49% amino acid sequence identity to the homologous protein of <i>P. putida</i> (Gcd, PP_1444), suggesting that the two proteins share comparable properties.

The kinetics of 5-KF production by <i>P. putida::fdhSCL</i> (<i>pBTT-inv1417</i>) from sucrose is comparable to that directly from fructose (Fig. 1) and even faster compared with <i>G. oxydans</i> with genomically integrated <i>fdhSCL</i> genes and plasmid-based <i>inv1417</i> expression (Hoffmann et al., 2020). This is surprising as Inv1417 is expected to be more active in <i>G. oxydans</i> at the beginning of the cultivations due to the lower start pH of 6. A possible limitation might be a higher number of membrane-bound dehydrogenases in <i>G. oxydans</i> compared with <i>P. putida</i>, which might limit the secretion of Fdh and invertase. A <i>G. oxydans</i> multideletion strain lacking eight membrane-bound dehydrogenases showed an increased l-erythrulose production by the native membrane-bound polyol dehydrogenase SldAB compared with the parental wild-type strain, which might be due at least in part to an improved Sec-dependent secretion of SldA (Peters et al., 2013; Burger et al., 2019). In summary, the results shown in Fig. 4 demonstrate that plasmid-based synthesis of the invertase Inv1417 from <i>G. japonicus</i> enables good growth of <i>P. putida::fdhSCL</i> with sucrose and efficient 5-KF production.

We also determined the specific invertase activity in cell-free extracts of <i>P. putida::fdhSCL</i> (<i>pBTT-inv1417</i>) and the control strain <i>P. putida::fdhSCL</i> (<i>pBTT</i>) by measuring sucrose consumption via HPLC. In extracts containing Inv1417, a specific activity of
4.63 ± 0.23 µmol min⁻¹ (mg protein)⁻¹ was measured, and similar rates were determined for the formation of the products glucose (4.38 ± 0.18 µmol min⁻¹ (mg protein)⁻¹) and fructose (4.03 ± 0.26 µmol min⁻¹ (mg protein)⁻¹). In contrast, no sucrose consumption was determined in extracts of the control strain without invertase. This result confirms the functional expression of inv1417 in P. putida.

**Bioreactor cultivation of P. putida::fdhSCL (pBT-T–inv1417) with sucrose**

After demonstrating efficient 5-KF production from sucrose in shake flasks by P. putida::fdhSCL (pBT-T–inv1417), we wanted to test the performance of the strain at elevated substrate concentrations under controlled conditions in a bioreactor. Preliminary tests showed that an initial concentration of 285 g l⁻¹ sucrose (corresponding to 150 g l⁻¹ fructose) is growth-inhibitory, and we therefore started with an initial sucrose concentration of 150 g l⁻¹ and added two pulses of sucrose after 13 and 23 h (Fig. 5). A growth rate of 0.43 h⁻¹ was reached with sucrose as sole carbon source and within 46 h 121 g l⁻¹ 5-KF were produced. Taking into account the initial sucrose concentration, the two sucrose pulses, and sucrose loss caused by sampling, 358 g sucrose were available in total. Considering 5-KF loss during sampling, 144 g 5-KF were produced in total, corresponding to a mass yield of 0.40 g 5-KF per g sucrose consumed and a molar yield of 0.77 mol 5-KF per mol sucrose. The space-time yield was 2.74 g l⁻¹ h⁻¹. After the pH drop and especially after the sucrose pulses, fast sucrose cleavage and fast 5-KF production were observed. As in the shake flask experiment (Fig. 4), high amounts of glucose remained in the medium (150 g l⁻¹), likely because the low pH prevented further metabolism (see discussion above).

**Conclusions and outlook**

In this study, we demonstrated that P. putida is able to functionally synthesize the fructose dehydrogenase of G. japonicus, which includes a membrane-anchored trihaem cytochrome c and an FAD-containing subunit transported picketpack via a small subunit via the Tat secretion system. The recombinant strain with genomically integrated fdhSCL genes proved to be an efficient 5-KF producer with comparable performance as G. oxydans, the standard host used for periplasmic oxidation reactions. By functional expression of the periplasmic invertase gene inv1417 from G. japonicus, we enabled growth of P. putida::fdhSCL on sucrose and 5-KF production from this substrate. Periplasmic conversion of sucrose to glucose and fructose is particularly reasonable for 5-KF production, as the substrate is formed directly in the compartment where it is needed (Fig. 6). Remarkably, the 5-KF production rate from sucrose was comparable to the one obtained directly with fructose, confirming that Inv1417 is a highly active invertase. Furthermore, our results indicate that P. putida has a high potential to serve as host for periplasmic oxidations not only with native enzymes, as recently published (Dvorák et al., 2020), but also with complex heterologous enzymes like G. japonicus Fdh.

Strategies to further optimize P. putida as host for 5-KF production can be envisaged. In G. oxydans the chromosomal integration of a second fdhSCL copy significantly increased the 5-KF production rate (Battling et al., 2020) and a comparable effect might also occur in P. putida with an additional chromosomal copy. Alternatively, plasmid-based expression of fdhSCL without the requirement for antibiotics selection could be used to increase the 5-KF production rate. Various antibiotic-free plasmid addiction systems have been described for bacteria (Kroll et al., 2010). For example, pyrF (encoding orotidine-5'-phosphate decarboxylase) and proC (encoding pyrroline-5-carboxylate reductase) were shown to be suitable plasmid selection markers in ΔpyrF and ΔproC strains of Pseudomonas fluorescens (Schneider et al., 2005). Furthermore, chromosomal expression of inv1417 or the use of an antibiotic-independent inv1417 expression plasmid would allow 5 KF production from sucrose without the necessity to use antibiotics. Lastly, process optimization will be important to improve 5 KF production.
with the strains generated in this study. For isolation of 5-KF, a method has been described in US patent 3,206,375, which involves removal of the cells, treatment of the supernatant with activated carbon for decolourization, deionization with ion exchange resins, concentration, precipitation and recrystallization (Kinoshita and Terada, 1963).

**Experimental procedures**

**Strains, plasmids and oligonucleotides**

All strains and plasmids used in this study are listed in Table 2. Plasmids were cloned in either *E. coli* DH5α or *E. coli* PIR2 (for the integration plasmid with ori R6K) via standard Gibson assembly (Gibson, 2011). Oligonucleotides used are listed in Table S1 and were synthesized by Eurofins Genomics (Ebersberg, Germany).

**Media composition and cultivation conditions**

*E. coli* strains and *P. putida* precultures were cultivated in LB medium (Bertani, 1951) at 37°C and 130 rpm or at 30°C and 180 rpm respectively. 5-KF production experiments with *P. putida* were conducted at 30°C, 85% humidity and 180 rpm at a shaking diameter of 50 mm in a Kuhner shaker ISF1-X (Kuhner, Birsfelden, Switzerland) using mineral salts medium (MSM) based on Hartmans et al. (1989). It contains per l double-distilled H2O 3.88 g K2HPO4, 1.63 g NaH2PO4 × 2 H2O, 2.0 g (NH4)2SO4, 0.1 g MgCl2 × 6 H2O, 10 mg EDTA, 2 mg ZnSO4 × 7 H2O, 1 mg CaCl2 × 2 H2O, 5 mg FeSO4 × 7 H2O, 0.2 mg Na3MoO4 × 2 H2O, 0.2 mg CuSO4 × 5 H2O, 0.4 mg CoCl2 × 6 H2O and 1 mg MnCl2 × 2 H2O with varying carbon sources at the indicated concentrations. When required, 50 µg ml⁻¹ kanamycin or 25 µg ml⁻¹ gentamycin were added. *G. oxydans* strains were cultivated at 30°C and 180 r.p.m in complex medium containing 40 g l⁻¹ mannitol, 5 g l⁻¹ yeast extract (BD Biosciences, Heidelberg, Germany), 2.5 g l⁻¹ MgSO4 × 7 H2O, 1 g l⁻¹ (NH4)2SO4 and 1 g l⁻¹ KH2PO4. The initial pH was adjusted to pH 6 with NaOH (Richhardt et al., 2013). The medium was supplemented with 50 µg ml⁻¹ cefoxitin and 10 µM thymidine.

### Generation of P. putida::fdhSCL via Tn7 integration

The Tn7-based chromosomal integration of the *fdhSCL* genes from *G. japonicus* was performed according to Zobel et al. (2015). **ATGfdhSCL** was amplified from pBBR1p264-fdhSCL-ST (Siemen et al., 2018) and encodes an FdhS variant with an ATG start codon instead of the original TTG start codon, which was shown to be advantageous (Kawai et al., 2013). Except for the start codon change, the native *G. japonicus* sequence was used. **fdhSCL** was cloned into pBG14g, with the Tn7L and Tn7R extremes, the left and right ends of Tn7 and the strong synthetic 14g promoter (Zobel et al., 2015). The resulting strain *E. coli* PIR2 pBG14g-fdhSCL was used for mating on LB agar with
the *P. putida* KT2440 wild-type recipient, the helper strain *E. coli* HB101 pRK2013 and *E. coli* DH5x λpir pTnS1, carrying the TnSABC-D operon, the TnSABC-D operon (Choi et al., 2005) according to Wynands et al. (2018). *P. putida* integration strains were selected on cetrime agar with gentamycin and correct integration downstream of the *glmS* gene (PP_5409) was confirmed via colony-PCR.

**Cultivation in 2-l bioreactor**

Bioreactor cultivations were conducted in a 2 l DASGIP bioreactor. The 1 l main cultures in MSM pH 7 with 150 g l⁻¹ fructose and 20 mM glucose or MSM pH 6.5 with 150 g l⁻¹ sucrose were inoculated from overnight shake flask precultures in the main culture medium. To prevent foam formation, headspace gassing was used. The DO was controlled at 30% via a cascade of increasing stirrer speed from 500 to 1200 rpm, the flow rate from 60 to 90 s L⁻¹ and the oxygen concentration from 21 to 80% (vol/vol).

**Sugar quantification via HPLC**

Culture samples were centrifuged (15 min, 17 000 g) and the supernatants stored at −20°C. Thawed samples were diluted with deionized water, heated for 60 min at 60°C to prevent double peaks for 5-KF, which might be caused by the keto and gem-diol forms (Herweg et al., 2018), filtered and analysed via high performance liquid chromatography (HPLC) using a modification of a previously described method (Richhardt et al., 2012). 10 µL samples were analysed with an Agilent LC-1100 system using a Rezex RCM-Monosaccharide 300 × 7.8 mm column (Phenomenex, Aschaffenburg, Germany) equipped with a Carbo-Ca Guard Cartridge (Phenomenex) at 80°C with water as eluent at a flow rate of 0.6 mL min⁻¹. Sucrose, glucose, 5-KF and fructose were detected using a refractometer index detector at 35°C at retention times of 9.4, 11.3, 13.1 and 14.5 min respectively.

**Preparation of cell free extracts for enzyme activity assays**

Overnight cultures of the respective *P. putida* and *G. oxydans* strains were harvested (10 min, 5000 g, 4°C), washed once in ddH₂O (Fdh assay) or 100 mM potassium phosphate buffer pH 6 (invertase assay) and the cells were disrupted in a Precellys 24 homogenizer (Bertin, Frankfurt am Main, Germany). Cell lysates were centrifuged for 10 min at 16 000 g and 4°C and the supernatant was collected as cell-free extract for the enzyme activity assays. Protein concentrations were determined via a modified Bradford assay using the Coo Protein Assay (Uptima, Interchim, Montlucon Cedex, France).

**Enzyme assays**

Fructose dehydrogenase activity was measured in a spectrophotometric assay with potassium ferricyanide as artificial electron acceptor as described (Ameyama and Adachi, 1982). Briefly, 100 µL McIlvaine buffer pH 4.5, 20

### Table 2. Bacterial strains and plasmids used in this work.

| Strain or plasmid | Relevant characteristic | Source or reference |
|-------------------|-------------------------|---------------------|
| **Bacterial strains** |                         |                     |
| *E. coli* DH5x | F− endA1 φ80lacZAM15 Δ(lacZYA-argF)U169 recA1 relA1 hsdR17(k−/m−/r−) deoR supE44 thi-1 gyrA96 proA1 λ−, strain used for cloning | Hanahan (1983) |
| PIR2 | F− Δlac169 rpoS(Am) rbaA1 creC510 hsdR514 endA recA1 uidA(l::Mud)λpir, host for ori R6K replication | Invitrogen |
| HB101 | F− mcrB mrr hsdS20(r−/m−B−) recA13 leuB6 ara−14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20(SmR) glm V44 λ− | Boyer and Roulland-Dussoix (1969) |
| DH5x λpir | λpir phage doxogen DH5s derivative; host for ori R6K vectors | Platt et al. (2000) |
| *P. putida* KT2440 | Wild-type, mt-2 derivative cured from plasmid pWW0 | Bagdasarian et al. (1981) |
| *P. putida::fdhSCL* | KT2440 with fdhSCL genes under control of P14g, integrated in attTn7, downstream of glmS | This study |
| **Plasmids** |                         |                     |
| pBG14g-msfgfp | KanR, GmR, ori R6K, Tn7L and Tn7R extremes, BCD2− msfp fusion, P14g | Zobel et al. (2015) |
| pBG14g-fdhSCL | pBG14g derivative for integration of fdhSCL | This study |
| pTnS1 | AmpR, ori R6K, TnSABC-D operon | Choi et al. (2005) |
| pRK2013 | KanR, ori (RK2/ColE1), mob tra | Figurski and Helinski (1979) |
| pBTmcs | KanR, ori and rep of pBBR1, Plac | Koopman et al. (2010) |
| pBBT | pBBTmcs with ribosomal binding site | Wiercx lab, Forschungszentrum Jülich, Germany |
| pBBT-inv1417 | pBBT derivative for expression of inv1417 | This study |
µl H2O, 20 µl 10% Triton X-100, 20 µl 1 M fructose and 20 µl cell-free extract were preincubated for 5 min at 25°C and then the reaction was started by adding 20 µl 0.1 M K3[Fe(CN)6]. Samples were incubated for 4-14 min at 25°C, the reaction was stopped by adding 100 µl ferric sulfate-Dupanol reagent (5 g L−1 Fe2((SO)4)3, 3 g L−1 sodium dodecyl sulphate, 95 ml L−1 85% phosphoric acid). 700 µl ddH2O was added and the samples were incubated for 20 min at 25°C. The formation of Prussian blue colour was measured at 660 nm. The kinetics of the absorbance increase was used to calculate the specific activity. One unit of enzyme activity is defined as the amount of enzyme catalysing the oxidation of 1 µmol D-fructose per minute under the conditions described above; 4.0 absorbance units equal 1 µmol of D-fructose oxidized (Ameaya and Adachi, 1982).

Invertase activity was measured according to a previously published method (Hoffmann et al., 2020) with slight modifications. 100 µl cell-free extract was mixed with 400 µl 100 mM potassium phosphate buffer pH 6 containing 1 M sucrose. The mixtures were incubated at 30°C and samples were taken at different time points after 20–120 min and stored at −20°C. Thawed samples were diluted with ddH2O and the sucrose, glucose and fructose concentrations were determined via HPLC. The specific activity was determined from the sucrose decrease over time, with one unit of enzyme activity corresponding to 1 µmol sucrose converted per minute.

Acknowledgements
The authors are very grateful for the support by their colleagues Prof. Nick Wierckx, Dr. Maike Otto and Dr. Benedikt Wynands, who provided Pseudomonas putida KT2440 and the plasmid vectors and gave advice with respect to cultivation and genetic engineering of P. putida. The invertase gene inv1417 from Gluconobacter japonicus was a kind gift from Prof. Uwe Deppenmeier and Dr. Juliane Hoffmann from the Institute of Microbiology and Biotechnology of the University of Bonn. The authors thank Rebecca Schößer for her contribution to this project during a practical course.

Funding Information
This project was funded by the Bundesministerium für Bildung und Forschung (BMBF) within the project IMPRES (FKZ 031B0370).

Conflict of interest
None declared.

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Supporting information

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

Fig. S1. Analysis of 5-KF formation by HPLC. In panel A, a standard mixture containing 5 g/L glucose, 5-KF and fructose was used. Fructose, glucose and 5-KF concentrations in the culture supernatants were determined by HPLC.

Fig. S2. Growth, pH, sugar consumption and 5-KF formation of P. putida::fdhSCL in batch cultivation. The strain was cultivated in 1 L MSM with 150 g/L fructose and 3.6 g/L glucose in a DASGIP bioreactor at 30 °C and DO ≥ 30%. After the initial acidification phase, the pH-control kept the pH at 5 by KOH addition. For inoculation, overnight shake flask precultures in MSM with 150 g/L fructose and 3.6 g/L glucose were used. Fructose, glucose, and 5-KF concentrations in the culture supernatants were determined by HPLC. The data for all four biological replicates performed in this study are shown. Panel D is identical to Fig. 2 in the main text.

Fig. S3. Growth, pH, sucrose and glucose consumption of P. putida::fdhSCL (pBT’-inv1417) in shake flasks. The strain was cultivated in 50 mL MSM with 100 mM sucrose.
in 500 mL shake flasks at 30 °C, 85% humidity, and 180 rpm (shaking diameter of 50 mm). The cultures were grown either under standard conditions with an initial pH of 7.0, or under the same condition, but with a pH shift after 24 h to pH 6.5 by addition of NaOH, or in modified MSM containing 72 mM phosphate buffer instead of 36 mM with an initial pH of 7, or in standard MSM with an initial pH of 6.5 All cultures were inoculated with precultures grown in MSM with 100 mM sucrose and 20 mM glucose, initial pH 7.0. Sucrose and glucose concentrations in the supernatants were determined by HPLC. Mean values and standard deviations of biological triplicates are shown.

**Fig. S4.** Growth, pH, sugar consumption and 5-KF formation of *P. putida::fdhSCL* (pBT’T-inv1417) in a bioreactor cultivation with pulsed feeding of sucrose. The strain was cultivated in 1 L MSM with 150 g/L sucrose in a DASGIP bioreactor at 30 °C and DO ≥ 30%. After the initial acidification phase, the pH was kept at pH 5 by addition of KOH. For inoculation, overnight shake flask precultures in MSM with 150 g/L sucrose were used. Additional sucrose was added as pulses of 100 mL and 165 mL of an 839 g/L sucrose solution after 13 and 23 h of cultivation, respectively (indicated by dashed lines). Sucrose, fructose, glucose, and 5-KF concentrations in the culture supernatants were determined by HPLC. The data for two biological replicates performed in this study are shown. Panel A is identical to Fig. 5 in the main text.

**Table S1.** Oligonucleotides used in this study.