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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,[1] Astrid Wirtz,[1] Alexander Reiter,[1,2] Marco Oldiges,[1,2] Meike Baumgart[1] and Michael Bott[1,3]*

[1]*IBG-1: Biotechnology, Institute of Bio- and Geosciences, Forschungszentrum Jülich, Jülich, 52425, Germany.*

[2]*Institute of Biotechnology, RWTH Aachen University, Aachen, 52062, Germany.*

[3]*The 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 *c* 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.

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*For correspondence. E-mail m.bott@fz-juelich.de; Tel. (+49) 2461 613294; Fax (+49) 2461 612710.
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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; Carocho *et al*., 2017). Reduction of added sugar consumption can be supported with non-nutritive sweeteners (Acero *et al*., 2020). As such, 5-ketofuctose (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 CXXCH, 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 pickaback 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

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 $g_{5\text{-KF}}/g_{fructose}$ 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 (Kiefler *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 (Kallnik *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 PQQ-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 CO_2. 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 bo_3 and a cyanide-insensitive *bd*-type oxidase ClO), a cytochrome bc_1 complex and three cytochrome *c* oxidases (cytochrome aa_3 and two cytochrome cbb_3 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; Kiefler *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 PTS^{Fru} composed of the two fusion proteins FruA (EIIB-EIIC) and FruB (EI-HPr-EIIA). 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 sucrose 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 Tn*7* 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 Tn*7* 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 $\pm$ 4 mM) when grown either on fructose alone or with 20 mM glucose as additional carbon source (22 $\pm$ 4 mM) and did not form 5-KF. *P. putida*::*fdhSCL* consumed the entire fructose within 48 h of cultivation and formed 57 $\pm$ 4 mM 5-KF when cultivated with fructose alone and 67 $\pm$ 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 $\pm$ 0.02 in medium with only fructose and 0.68 $\pm$ 0.03 in glucose-supplemented medium. Although the molar yield related to the sum of fructose and glucose consumed (0.56 $\pm$ 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 $\pm$ 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 $\pm$ 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

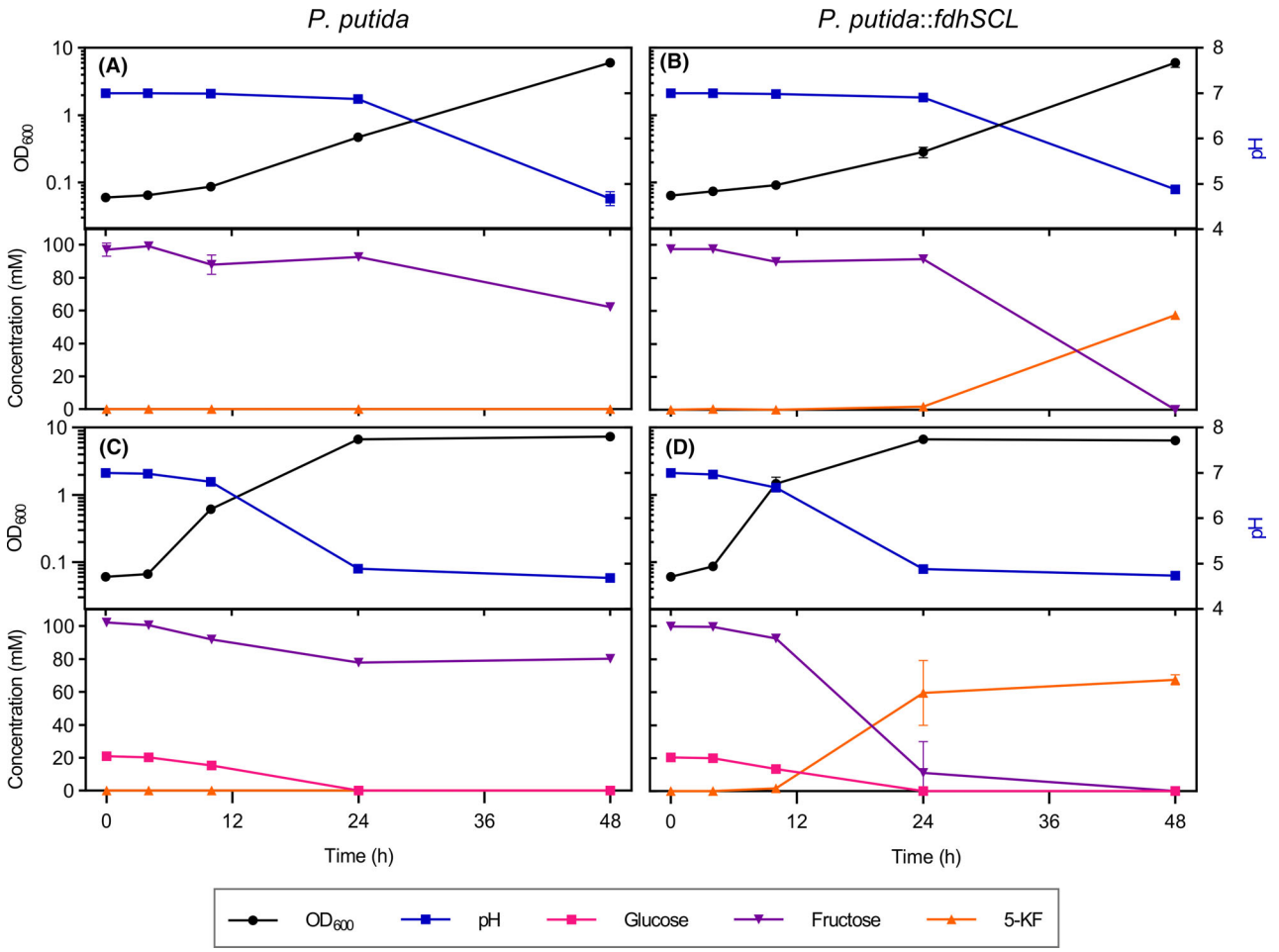

Fig. 1. Growth, pH, sugar consumption and 5-KF formation of *P. putida* and *P. putida::fdhSCL* in shake flasks. *P. putida* wild type (A, C) and *P. putida::fdhSCL* (B, D) were cultivated in 50 ml MSM with 100 mM fructose only (A, B) and MSM with 100 mM fructose and 20 mM glucose (C, D) in 500 ml shake flasks at 30 °C, 85% humidity, and 180 rpm with a shaking diameter of 50 mm. Glucose, fructose and 5-KF concentrations in the culture supernatant were determined by HPLC. Mean values and standard deviations of biological triplicates are shown.

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 OD_{600} = 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

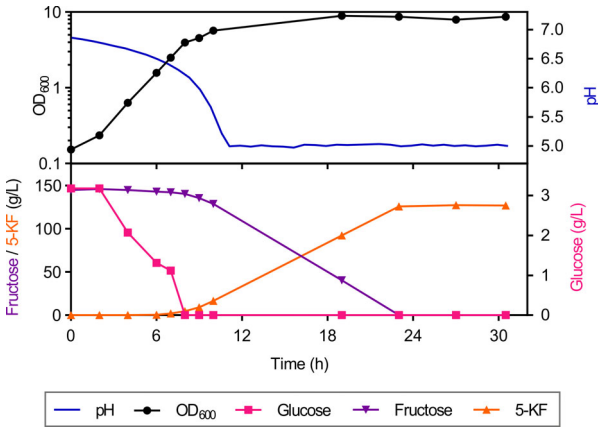

Fig. 2. Growth, pH, sugar consumption and 5-KF formation of *P. putida::fdhSCL* in a batch cultivation. The strain was cultivated in 1 l MSM with 150 g l^{-1} fructose and 3.6 g l^{-1} glucose in a DASGIP bioreactor at 30°C and DO ≥ 30%. After the initial acidification phase, the pH was kept at pH 5 by addition of KOH. For inoculation, overnight shake flask 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*[a]	*P. putida*::*fdhSCL*[b]
μ (h^{-1})	n.d.	0.48 ± 0.02
Final OD_{600}	9.2	8.55 ± 0.36
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

a. Data for *G. oxydans* IK003.1-igr3::*fdhSCL* cultivated in complex medium with 150 g l^{-1} fructose were taken from Battling *et al.* (2020).
b. Mean values and standard deviations from four biological replicates of *P. putida*::*fdhSCL* in MSM with 150 g l^{-1} fructose and 3.6 g l^{-1} glucose.

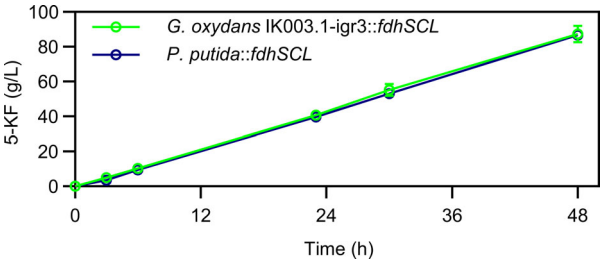

Fig. 3. Biotransformation of fructose to 5-KF with resting cells. Cell suspensions of *G. oxydans* IK003.1-igr3::*fdhSCL* and *P. putida*::*fdhSCL* at an OD_{600} of 3 were incubated in 100 mM potassium phosphate buffer pH 6 with 150 g l^{-1} fructose at 30°C and 180 rpm. 5-KF concentrations were determined by HPLC. Mean values and standard deviations of biological triplicates are shown.

of incubation. However, when these cells were sedimented by centrifugation and used for a second biotransformation, the 5-KF production rate was much lower, indicating that repeated use of the cells is not an option (data not shown).

Expansion 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; Löwe *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 K_M 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_{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.

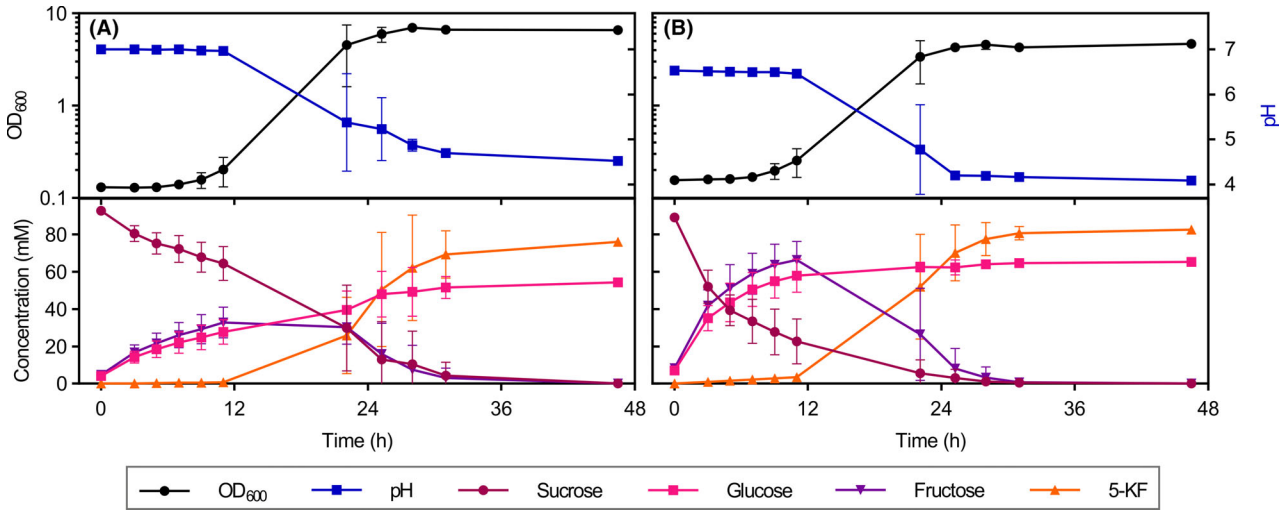

Fig. 4. Growth, pH, sugar consumption and 5-KF formation of *P. putida*::*fdhSCL* (pBT'T-*inv1417*) in shake flasks. The strain was cultivated in 50 mL MSM with 100 mM sucrose in 500 ml shake flasks at 30 °C, 85% humidity, and 180 rpm (shaking diameter of 50 mm) with a start pH of either 7.0 (A) or 6.5 (B). The cultures were inoculated with precultures grown in MSM with 100 mM sucrose and 20 mM glucose. Sucrose, glucose, fructose and 5-KF concentrations in the supernatants were determined by HPLC. Mean values and standard deviations of biological triplicates are shown.

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_{600} values (Fig. S3). The major pathway for glucose catabolism in *P. putida* 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 *P. putida*, the pH optimum has not been determined to our knowledge. However, in recent studies on lactobionic acid production with *Pseudomonas taetrolens* 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 *P. taetrolens* 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 *P. taetrolens* (GenBank: KMM82267.1) shows 49% amino acid sequence identity to the homologous protein of *P. putida* (Gcd, PP_1444), suggesting that the two proteins share comparable properties.

The kinetics of 5-KF production by *P. putida*::*fdhSCL* (pBT'T-*inv1417*) from sucrose is comparable to that directly from fructose (Fig. 1) and even faster compared with *G. oxydans* with genomically integrated *fdhSCL* genes and plasmid-based *inv1417* expression (Hoffmann *et al.*, 2020). This is surprising as Inv1417 is expected to be more active in *G. oxydans* 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 *G. oxydans* compared with *P. putida*, which might limit the secretion of Fdh and invertase. A *G. oxydans* 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 *G. japonicus* enables good growth of *P. putida*::*fdhSCL* with sucrose and efficient 5-KF production.

We also determined the specific invertase activity in cell-free extracts of *P. putida*::*fdhSCL* (pBT'T-*inv1417*) and the control strain *P. putida*::*fdhSCL* (pBT'T) 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 metabolization (see discussion above).

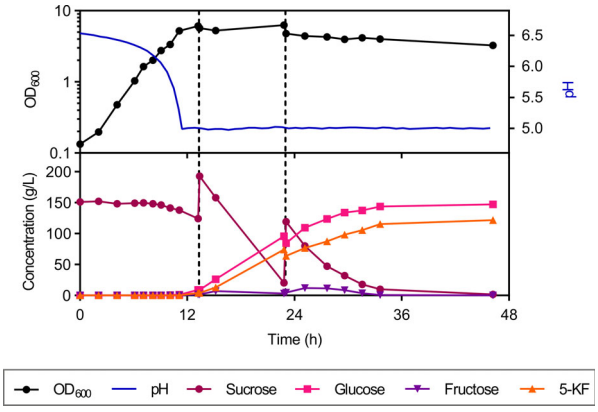

Fig. 5. Growth, pH, sugar consumption and 5-KF formation of *P. putida::fdhSCL* (pBT'T-*inv1417)* in a bioreactor cultivation with pulsed feeding of sucrose. The strain was cultivated in 1 l MSM with 150 g l^{-1} sucrose in a DASGIP bioreactor at 30°C, DO ≥ 30% and pH control at pH 5 by KOH addition after the initial acidification phase. For inoculation overnight shake flask precultures in MSM with 150 g l^{-1} sucrose were used. Additional sucrose was added as pulses of 100 ml and 165 ml of an 839 g l^{-1} 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. A representative example of two biological replicates (Fig. S4) is shown here.

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 pickepack with 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 (Dvořák *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 Δ*pyrF* and Δ*proC* 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

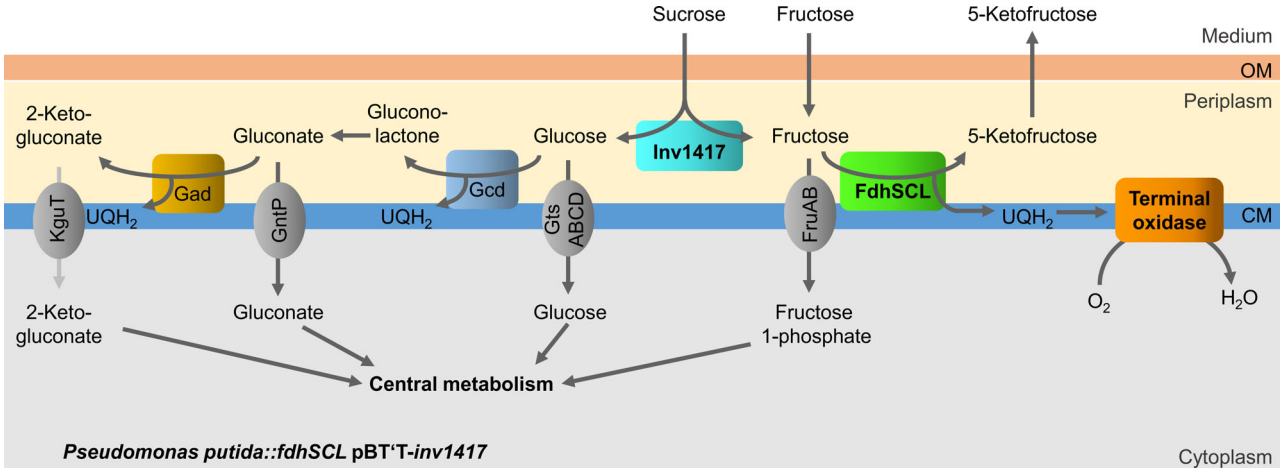

Fig. 6. Schematic overview of sugar metabolism and 5-KF production in *P. putida::fdhSCL* (pBT'T-*inv1417*). Sucrose and fructose enter the periplasm via porins (not shown) in the outer membrane (OM). Sucrose is cleaved by the periplasmic invertase Inv1417 of *G. japonicus* to glucose and fructose. Fructose is oxidized to 5-ketofructose by the membrane-bound fructose dehydrogenase FdhSCL from *G. japonicus*. Glucose can be taken up into the cytosol via the cytoplasmic membrane (CM) by the glucose transport system (GtsABCD) or is oxidized by the PQQ-dependent glucose dehydrogenase (Gcd) to gluconolactone, which is subsequently hydrolyzed to gluconate. Gluconate can be taken up via the gluconate permease (GntP) or is further oxidized by the gluconate dehydrogenase (Gad) to 2-ketogluconate, which can be taken up via the ketogluconate transporter (KguT). Periplasmic oxidation by the membrane-bound dehydrogenases generates ubiquinol, which is oxidized in the respiratory chain either directly by ubiquinol oxidases or via the cytochrome bc_1 complex and cytochrome *c* oxidases.

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 H_2O 3.88 g K_2HPO_4, 1.63 g $NaH_2PO_4 \times 2$ H_2O, 2.0 g $(NH_4)_2SO_4$, 0.1 g $MgCl_2 \times 6$ H_2O, 10 mg EDTA, 2 mg $ZnSO4 \times 7$ H_2O, 1 mg $CaCl_2 \times 2$ H_2O, 5 mg $FeSO_4 \times 7$ H_2O, 0.2 mg $Na_2MoO_4 \times 2$ H_2O, 0.2 mg $CuSO_4 \times 5$ H_2O, 0.4 mg $CoCl_2 \times 6$ H_2O and 1 mg $MnCl_2 \times 2$ H_2O 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} $MgSO_4 \times 7$ H_2O, 1 g l^{-1} $(NH_4)_2SO_4$ and 1 g l^{-1} KH_2PO_4. 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 Tn*7*-based chromosomal integration of the *fdhSCL* genes from *G. japonicus* was performed according to Zobel *et al.* (2015). $_{ATG}$*fdhSCL* 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 Tn*7*L and Tn*7*R extremes, the left and right ends of Tn*7* and the strong synthetic 14g promotor (Zobel *et al.*, 2015). The resulting strain *E. coli* PIR2 pBG14g-*fdhSCL* was used for mating on LB agar with

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

Strain or plasmid	Relevant characteristic	Source or reference
Bacterial strains		
E. coli		
DH5α	*F⁻ endA1* Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 relA1 hsdR17*(rK–mK+) *deoR supE44 thi-1 gyrA96 phoA* λ–, strain used for cloning	Hanahan (1983)
PIR2	*F⁻ Δlac169 rpoS(Am) robA1 creC510 hsdR514 endA reacA1 uidA(ΔMlui)::pir*, host for ori R6K replication	Invitrogen
HB101	*F⁻ mcrB mrr hsdS20(rB– mB –) recA13 leuB6 ara–14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20(SmR) gln V44* λ–	Boyer and Roulland-Dussoix (1969)
DH5α λpir	λpir phage lysogen DH5α 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 *att*Tn*7*, downstream of *glmS*	This study
Plasmids		
pBG14g-*msfgfp*	Kan^R, Gm^R, ori R6K, Tn*7*L and Tn*7*R extremes, BCD2–*msfgfp* fusion, P_{14g}	Zobel *et al.* (2015)
pBG14g-*fdhSCL*	pBG14g derivative for integration of *fdhSCL*	This study
pTnS1	Amp^R, ori R6K, TnSABC+D operon	Choi *et al.* (2005)
pRK2013	Kan^R, oriV(RK2/ColE1), mob^+ tra^+	Figurski and Helinski (1979)
pBT'mcs	Kan^R, ori and rep of pBBR1, P_{tac}	Koopman *et al.* (2010)
pBT'T	pBT'mcs with ribosomal binding site	Wierckx lab, Forschungszentrum Jülich, Germany
pBT'T-*inv1417*	pBT'T derivative for expression of *inv1417*	This study

the *P. putida* KT2440 wild-type recipient, the helper strain *E. coli* HB101 pRK2013 and *E. coli* DH5α λpir pTnS1, carrying the TnS_{ABC+D} operon for site-directed transposition (Choi *et al.*, 2005) according to Wynands *et al.* (2018). *P. putida* integration strains were selected on cetrimide 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 the stirrer speed from 500 to 1200 rpm, the flow rate from 60 to 90 sL h^{-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 refraction 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 ddH_2O (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

μl H_2O, 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 $K_3[FeIII(CN)_6]$. 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} $Fe_2((SO)_4)_3$, 3 g l^{-1} sodium dodecyl sulphate, 95 ml l^{-1} 85% phosphoric acid). 700 μl ddH_2O 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 (Ameyama 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 ddH_2O 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.

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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 separated. In panel B, supernatants (10 μl) of a shake flask cultivation of *P. putida*::*fdhSCL* (see Fig. 1) at the start of the cultivation (grey) and after 48 h (black) were analyzed with an Agilent LC-1100 system using a Rezex RCM-Monosaccharide 300 x 7.8 mm column (Phenomenex, Aschaffenburg, Germany) equipped with a Carbo-Ca Guard Cartridge (Phenomenex, Aschaffenburg, Germany) at 80 °C with water as eluent at a flow rate of 0.6 mL/min. A refraction index detector operated at 35 °C was used for detection.

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'T-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 $\geq$ 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.

