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Genetic engineering of *Pseudomonas putida* KT2440 for rapid and high-yield production of vanillin from ferulic acid

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Abstract Vanillin is one of the most important flavoring agents used today. That is why many efforts have been made on biotechnological production from natural abundant substrates. In this work, the nonpathogenic *Pseudomonas putida* strain KT2440 was genetically optimized to convert ferulic acid to vanillin. Deletion of the vanillin dehydrogenase gene (*vdh*) was not sufficiant to prevent vanillin degradation. Additional inactivation of a molybdate transporter, identified by transposon mutagenesis, led to a strain incapable to grow on vanillin as sole carbon source. The bioconversion was optimized by enhanced chromosomal expression of the structural genes for feruloyl-CoA synthetase (*fcs*) and enoyl-CoA hydratase/aldolase (*ech*) by introduction of the strong *tac* promoter system. Further genetic engineering led to high initial conversion rates and molar vanillin yields up to 86 % within just 3 h accompanied with very low by-product levels. To our knowledge, this represents the highest productivity and molar vanillin yield gained with a *Pseudomonas* strain so far. Together with its high tolerance for ferulic acid, the developed, plasmid-free *P. putida* strain represents a promising candidate for the biotechnological production of vanillin.

Keywords Bioconversion · *Pseudomonas putida* · Vanillin · Ferulic acid · Genetic engineering · Plasmid-free

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Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde), the organoleptic compound of the vanilla flavor, is one of the quantitative most widely used flavoring agents worldwide. Its demand has long exceeded the supply by the botanical source *Vanilla planifolia*. At present, most of the vanillin is synthesized chemically from guaiacol, which originates from fossile raw materials, and lignin, a component in waste material from the wood pulp industry (Ramachandra Rao and Ravishankar 2000). However, the demand for this "nature-identical" vanillin, which is mostly used in the food and beverage industry, is shifted towards the "natural" vanillin due to a rising health- and nutrition consciousness of the costumers. Thus, biotechnological production of natural vanillin becomes more and more important (reviewed by Krings and Berger 1998; Priefert et al. 2001).

Efforts have been made to produce vanillin by in vitro cultured *V. planifolia* cells (Davidonis and Knorr 1991). A de novo synthesis was also implemented using genetically modified yeast strains (Hansen et al. 2009). The main focus, however, was put on the biotransformation using isolated enzymes or different prokaryotic microorganisms as whole cell biocatalysts (Havkin-Frenkel and Belanger 2008; Berger 2009).

Besides lignin and phenolic stilbenes, like eugenol, the biotransformation of ferulic acid to vanillin is the most intensively studied method to produce "natural" vanillin (reviewed by Rosazza et al. 1995; Priefert et al. 2001). The precursor ferulic acid (3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid), a hydroxycinnamic acid, is a highly abundant substance since it is a constituent of many plant cell walls (Ishikawa et al. 1963; Escott-Watson and Marais 1992; Ishii 1997; Oosterveld et al. 2000). Many different microorganisms have been evaluated for the production of vanillin from ferulic acid

comprising recombinant strains of *Escherichia coli*, *Pseudomonas* ssp., *Rhodococcus* ssp., *Bacillus subtilis*, *Aspergillus niger*, *Pycnoporus cinnabarinus*, *Amycolatopsis* ssp., and *Streptomyces* ssp. (Lesage-Meessen et al. 1996; Okeke and Venturi 1999; Muheim and Lerch 1999; Achterholt et al. 2000; Overhage et al. 2003; Peng et al. 2003; Plaggenborg et al. 2006; Yoon et al. 2007; Barghini et al. 2007; Hua et al. 2007; Di Gioia et al. 2010; Tilay et al. 2010; Fleige et al. 2013). However, in most cases, vanillin yields were low and biotransformation reactions slow. The low yields can mostly be ascribed to the high toxicity of vanillin (Krings and Berger 1998). Enhanced vanillin production with adsorbent resins improved the vanillin levels up to 19.2 g l^{-1}, but the molar yield of about 43 % was rather low (Hua et al. 2007). Other drawbacks were inefficient heterologous gene expression and plasmid instabilities. A focus was also set on prevention of further degradation of vanillin to vanillyl alcohol or vanillic acid (Stentelaire et al. 1997; Bonnin et al. 1999; Oddou et al. 1999; Civolani et al. 2000; Overhage et al. 2000).

Bacteria from the genus *Pseudomonas* show a broad metabolic versatility as they can use a wide range of aromatic molecules as sole carbon sources (Clarke 1982). The ferulic acid catabolism in *Pseudomonas* sp. strain HR199, *Pseudomonas fluorescens* BF13, and *Pseudomonas putida* KT2440 occurs via a coenzyme A-dependent, non-β-oxidative pathway as depicted in Fig. 1 (Narbad and Gasson 1998; Gasson et al. 1998; Overhage et al. 1999b; Plaggenborg et al. 2003; Calisti et al. 2008). First, ferulic acid becomes activated to feruloyl-CoA catalyzed by feruloyl-CoA synthetase (EC 6.2.1.34; encoded by *fcs*). Second, the CoA thioester is hydrated and cleaved to vanillin and acetyl-CoA catalyzed by enoyl-CoA hydratase/aldolase (EC 4.2.1.101; encoded by *ech*). The vanillin dehydrogenase (EC 1.2.1.67; encoded by *vdh*) oxidizes vanillin to vanillic acid which is further catabolized to protocatechuic acid by vanillate-*O*-demethylase (EC 1.14.13.82; encoded by *vanAB*). Overhage et al. (1999b) also proposed a second route over 4-hydroxy-3-methoxyphenyl-β-ketopropionyl-CoA and vanillyl-CoA catalyzed by enzymes encoded by PP_3355 (*aat*) and probably PP_3354.

A recent study has used a metabolic engineered strain of *P. fluorescens* for the production of vanillin from ferulic acid (Di Gioia et al. 2010). By deletion of the gene *vdh* for the vanillin dehydrogenase and by overexpression of the structural genes *fcs* and *ech* on a low-copy vector, the authors were able to produce up to 8.41 mM vanillin from 10 mM ferulic acid which was the highest final titer of vanillin produced with a *Pseudomonas* strain so far.

In this study, we used the nonpathogenic, fully sequenced *P. putida* strain KT2440 (ATCC 47054) (Nelson et al. 2002), a plasmid-free derivative of the biosafety strain *P. putida* mt-2 (Kojima et al. 1967; Williams and Murray 1974; Nakazawa 2002). We describe a highly efficient way for the biotransformation of ferulic acid to vanillin using plasmid-free, resting *P. putida* mutant cells. Genetic manipulation of *P. putida* KT2440 using the *upp* counterselection system (Graf and Altenbuchner 2011) led to cells which were able to rapidly convert ferulic acid to vanillin accompanied with molar yields up to 86 %, high productivities, and only little by-product formation.

Materials and methods

Plasmids, bacterial strains, and growth conditions

The bacterial strains and plasmids used in this study are shown in Table 1. *P. putida* strains were grown at 30 °C and *E. coli* JM109 (Yanisch-Perron et al. 1985) at 37 °C in LB medium (Bertani 1951). For selection of plasmids 50 μg ml^{-1} kanamycin (Kan) was added. During the deletion procedure and for the tolerance tests, M9 minimal medium was used for growth of *P. putida* strains (48 mM $Na_2HPO_4 \cdot 7\ H_2O$, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl), supplemented with 0.2 % glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 6 μM thiamine hydrochloride, and 20 μg ml^{-1} 5-fluorouracil (5-FU; prepared as a stock solution of 100 mg ml^{-1} in dimethyl sulfoxide (DMSO)).

Chemicals and other materials

Chemicals used in this study were of analytical grade and purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany), Sigma-Aldrich Corporation (Taufkirchen, Germany), and Merck KGaA (Darmstadt, Germany). In particular, 5-FU, ferulic acid, vanillin, vanillic acid, and vanillyl alcohol were purchased from Sigma-Aldrich. Synthetic DNA oligonucleotides (Table S1) were purchased from Eurofins MWG Operon GmbH (Ebersberg, Germany). Restriction enzymes and DNA modifying enzymes were purchased from Roche Diagnostics Deutschland GmbH (Mannheim, Germany), New England Biolabs GmbH (Frankfurt am Main, Germany), and Fermentas GmbH (part of Thermo Fisher Scientific, St. Leon-Rot, Germany). PCRs were run with High Fidelity PCR Enzyme Mix from Fermentas GmbH on a TPersonal Thermocycler from Biometra GmbH (Göttingen, Germany).

Vector construction and genetic manipulation of *P. putida* strains

Cloning steps were performed with *E. coli* JM109 (Yanisch-Perron et al. 1985) using standard recombinant DNA techniques (Sambrook et al. 1989). Transformation of *E. coli* with plasmid DNA occurred via the Transformation and Storage

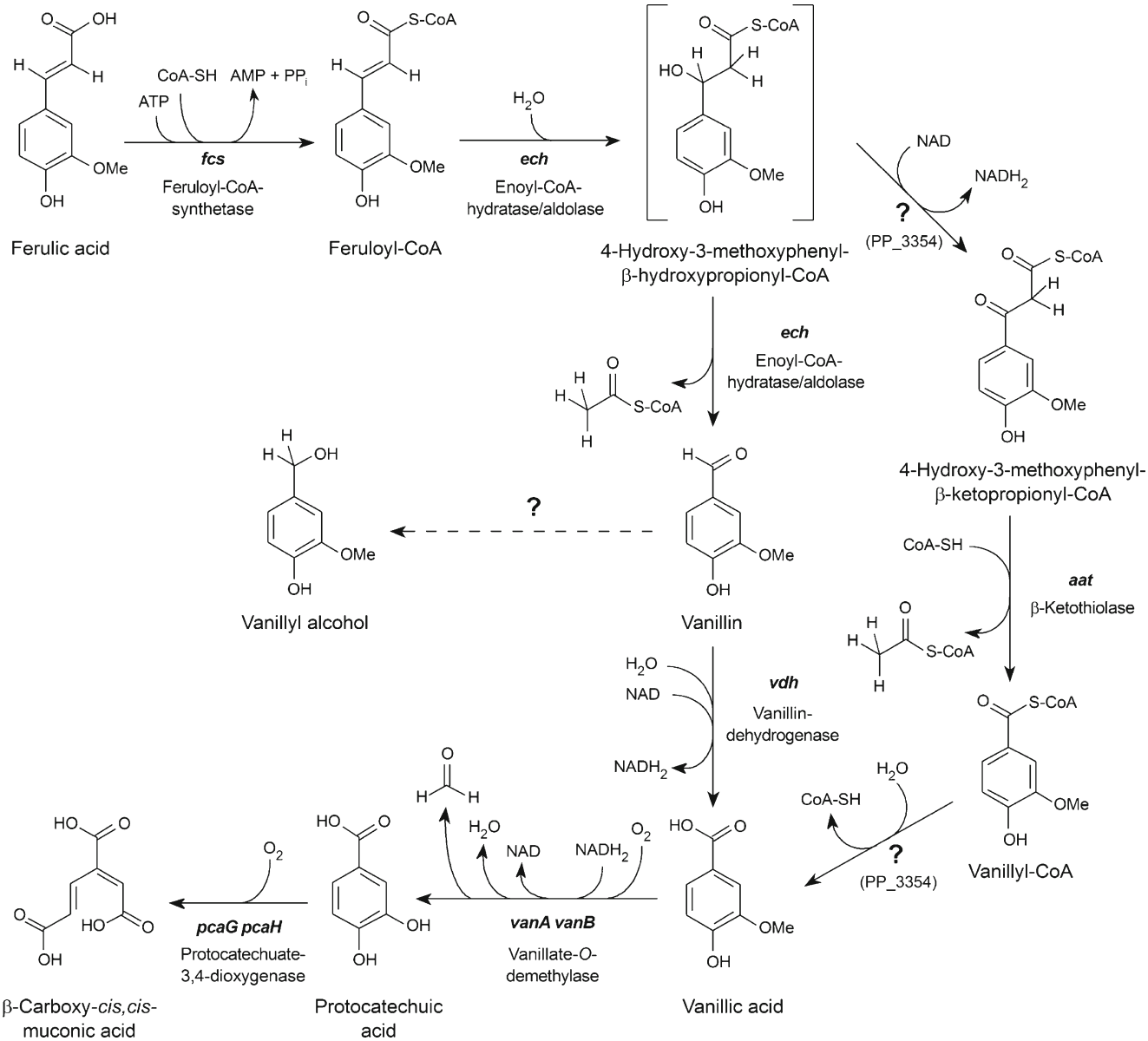


Fig. 1 Proposed route for the catabolism of ferulic acid over vanillin in *Pseudomonas* strains. The alternative route from 4-hydroxy-3-methoxyphenyl-β-hydroxypropionyl-CoA to vanillic acid is shown on the *right* (proposed by Overhage et al. 1999b). The reduction of vanillin to vanillyl alcohol is depicted by a *dashed arrow*. *Question marks* symbolize reactions catalyzed by unknown enzymes

Solution method (Chung et al. 1989). *P. putida* strains were transformed with plasmid DNA via electroporation (Sambrook et al. 1989). Construction of the plasmids and strains is summarized in Table S2.

For chromosomal deletions and integrations in *P. putida* KT2440, the *upp*/5-FU counterselection system was used as described previously (Graf and Altenbuchner 2011). First, the up- and downstream regions including the start and stop codons of the target gene were PCR-amplified using chromosomal DNA of *P. putida* KT2440 (GenBank accession number AE015451) as template. These fragments were cloned via 3-fragment ligation into pJOE6261.2. The resulting integration vector was then used for electroporation of *P. putida* ΔUPP4 or other *upp* deleted strains. One of the Kan^r 5-FU^s clones obtained was incubated in LB medium for 24 h at 30 °C under shaking conditions (200 rpm). Afterwards, different dilutions were plated on minimal plates containing 20 μg ml^{-1} 5-FU and 0.2 % glucose. Ten 5-FU^r and Kan^s clones were checked by colony PCR, using oligonucleotides binding to the up- and downstream sequences of the gene to be deleted.

Construction of the *lacI^q-P$_{tac}$* integration vector pNG283.5 started with the PCR amplification of *ech* using oligonucleotide primers s6936/s6937 and chromosomal DNA of *P. putida* KT2440 as template. The purified PCR fragment (897 bps) was cloned via *Nde*I/*Bam*HI into pJOE5304.1

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Reference or source
Strains		
E. coli		
JM109	*recA1, supE44, endA1, hsdR17, gyrA96, relA1, thi, Δ(lac-proAB), F' [traD36 proAB+ lacI^q lacZΔM15]*	Yanisch-Perron et al. (1985)
S17.1	*recA pro hsdR* RP4-2-*Tc::Mu-Km::*Tn7	Simon et al. (1983)
P. putida		
KT2440	wild type	ATCC 47054
ΔUPP4	Δ*upp*	Graf and Altenbuchner (2011)
GN23	Δ*upp* ΔPP_0166-0168	This study
GN235	Δ*upp* ΔPP_0166-0168 Δ*vdh*	This study
GN275	Δ*upp* ΔPP_0166-0168 Δ*vdh modA::*mini-Tn*5495*	This study
GN276	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832	This study
GN299	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832 *lacI^q-P$_{tac}$-ech-fcs*	This study
GN347	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832 ΔPP_3354 ΔPP_3355 *lacI^q-P$_{tac}$-ech-fcs*	This study
GN440	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832 ΔPP_2680 *lacI^q-P$_{tac}$-ech-fcs*	This study
GN441	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832 ΔPP_2680 ΔPP_0545 *lacI^q-P$_{tac}$-ech-fcs*	This study
GN442	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832 ΔPP_2680 ΔPP_0545 ΔPP_1948 *lacI^q-P$_{tac}$-ech-fcs*	This study
Plasmids		
pCro2a	mini-Tn*5495* delivery vector	Onaca et al. (2007)
pJOE5304.1	expression vector with *lacI^q*-P$_{tac}$-*eGFP*	laboratory stock
pJOE6261.2	pIC20HE (Altenbuchner et al. 1992) backbone with a kanamycin resistance gene and a copy of *upp* from *P. putida* KT2440	Graf and Altenbuchner (2011)
pNG53.1	pJOE6261.2 with the upstream region of PP_0166 and downstream region of PP_0168 cloned into *Bam*HI site	This study
pNG173.1	pJOE6261.2 with the up- and downstream regions of *vdh* cloned into *Bam*HI site	This study
pNG260.4	pJOE6261.2 with the upstream region of PP_3827 and downstream region of PP_3832 cloned into *Bam*HI site	This study
pNG276.1	pJOE6261.2 with the up- and downstream regions of PP_2680 cloned into *Sal*I site	This study
pNG281.1	pJOE5304.1 derivative with *lacI^q*-P$_{tac}$-*ech*	This study
pNG283.5	pJOE6261.2 with 900 bp of *ech*, *P$_{tac}$*, *lacI^q* and the downstream region of *ech* cloned into *Bam*HI site	This study
pNG338.1	pJOE6261.2 with the up- and downstream regions of PP_0545 cloned into *Bam*HI site	This study
pNG340.2	pJOE6261.2 with the upstream region of PP_3354 and downstream region of *aat* cloned into *Bam*HI site	This study
pNG412.1	pJOE6261.2 with the up- and downstream regions of PP_1948 cloned into *Bam*HI site	This study

resulting in pNG281.1, a vector with a *lacI^q*-P$_{tac}$-*ech* cassette. Next, this cassette was PCR-amplified with s6936/s6965 (fragment A; 2,376 bps). Also, the upstream region of *ech* was PCR-amplified with s6938/s6939 (fragment B; 952 bps). Fragments A and B were cut with *Bam*HI/*Mfe*I and *Eco*RI/*Bam*HI, respectively, and cloned via 3-fragment ligation into *Bam*HI cut pJOE6261.2, giving pNG283.5.

Mating and transposon mutagenesis

Overnight cultures of *E. coli* S17.1/pCro2a (contains mini-Tn*5495*) (Onaca et al. 2007) and *P. putida* GN235 grown in LB with and without kanamycin, respectively, were mixed equally (200 μl each) and 100 μl of that mixture was dropped onto a LB agar plate without antibiotics. After incubation for 24 h at 30 °C, grown cells were scraped off the plate with 3 ml LB liquid medium. In each case, 100 μl of a 10^{-2} dilution (giving about 50–100 colonies) was plated on a total of 50 LB agar plates containing 50 μM kanamycin and 10 μM nalidixid acid (Nal) (for counterselection of the *E. coli* donor). The plates were then incubated for 40 h at 30 °C. From each plate, the colonies were replica plated on M9 minimal agar plates, one with 0.2 % (*w*/*v*) glucose and the other one with 0.1 % (*w*/*v*) vanillin. Incubation occured overnight at 30 °C. Colonies which were grown on M9 plates with glucose but not on M9 plates with vanillin were toothpicked on a LB agar plate with 50 μM kanamycin and 10 μM nalidixid acid, on a M9 agar plate with 0.1 %

vanillin and on a M9 agar plate with 0.1 % vanillic acid and incubated overnight at 30 °C. The chromosomal DNA from clones which had grown on $LB_{Kan/Nal}$ and on M9 with vanillic acid but not on M9 with vanillin was isolated (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) and digested with restriction enzymes *Bsr*GI, *Eco*RI, and *Sal*I, respectively. The chromosomal fragments were purified (NucleoSpin Extract II Kit, Macherey-Nagel, Düren, Germany), ligated overnight at 4 °C, precipitated with isopropanol for 2 h on ice, washed with ethanol, and resuspended in 10 μl H_2O (bidest.). *E. coli* JM109 was transformed with the ligated chromosomal fragments. Selection occured on LB agar plates containing 50 μM kanamycin. Plasmids were isolated from kanamycin resistant clones and checked by restriction enzyme digestion. After sequencing of the plasmids with primers s4052 and s4037 (Onaca et al. 2007) (GATC Biotech, Constance, Germany), the obtained sequences were finally subjected to a BLAST search.

Bioconversion assay of ferulic acid to vanillin

Overnight cultures of *P. putida* strains were diluted 1:50 in fresh LB medium and grown for 2 h at 30 °C in shaking flasks (200 rpm). Induction of the ferulic acid metabolic genes occurred by addition of 5 mM ferulic acid or 5 mM IPTG depending on the strain. After further growth for 6 h at 30 °C under shaking conditions, 25×10^9 cells were harvested by centrifugation (10 min, 3,500×*g*, room temperature), washed, and resuspended with 5 ml of 50 mM sodium phosphate buffer (pH 7.2). A total of 10 mM of ferulic acid (1 M stock solution in DMSO) was added to the cell suspension. The bioconversion was conducted in long glass culture tubes at 30 °C under shaking conditions (200 rpm). Samples of 200 μl were taken after 1, 2, 3, 4, 5, and 18 h conversion time. After a centrifugation step (10 min, 16,000×*g*, room temperature) to pellet the cells, 100 μl of the supernatant was collected and stored at −70 °C until analysis through HPLC.

Analytical methods

Samples from the bioconversion assay were diluted 1:10 with 0.2 % acetic acid prior to HPLC application. Ferulic acid, vanillin, vanillic acid, and vanillyl alcohol were quantified with a Merck-Hitachi HPLC system (Merck, Darmstadt, Germany) equipped with a RP Purospher®-Star RP-18e column (250 mm×4.6 mm, 5 μm), a LiChroCART® guard column (4 mm×4 mm, 5 μm), an L7612 degasser, an L6200A gradient pump, a D6000A interface module, an L4200 UV–visible detector, a Rheodyne injection valve 7125 with a 100-μl sample loop, and D7000 HPLC System Manager software. For measurements, a modified procedure was used as described previously (Sinha et al. 2007): methanol, acetonitrile, and 0.2 % acetic acid (3:3:14) were used as the mobile phase. The flow rate was 1 ml min^{-1} and the absorbance was measured at 231 nm for 20 min. Solutions of ferulic acid, vanillin, vanillic acid, and vanillyl alcohol with seven different concentrations (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 1 mM) were used for calibration.

Results

Construction and characterization of a *P. putida* KT2440 mutant unable to grow on vanillin as sole carbon source

As reported previously (Overhage et al. 1999b; Plaggenborg et al. 2003), *P. putida* KT2440 is able to grow on ferulic acid as sole carbon source. Ferulic acid is metabolized in a few steps to vanillin, catalized by feruloyl-CoA-synthetase (PP_3356, *fcs*) and enoyl-CoA-hydratase/aldolase (PP_3358, *ech*). Vanillin in turn gets further degraded to vanillic acid by the vanillin dehydrogenase (PP_3357, *vdh*). The last step has to be prevented, if vanillin accumulation is desired. The chromosomal organization of these genes in *P. putida* KT2440 and other strains constructed in this study is shown in Fig. 2.

With respect to industrial applications, we constructed *P. putida* strain GN23 with a deletion in the *lapABC* operon including the gene for the surface adhesion protein (PP_0168, *lapA*) using the previously described *upp* counterselection method (Graf and Altenbuchner 2011). The surface adhesion protein is responsible and essential for the formation of biofilms as previously shown for another *P. putida* KT2440 Δ*lapA* mutant strain (Graf and Altenbuchner 2011).

In the second step, the chromosomal *vdh* gene of *P. putida* GN23 was deleted leaving just the start and stop codon of *vdh* (Fig. 2). The resulting strain, designated as GN235, was still able to grow on ferulic acid as sole carbon source demonstrating functional expression of *fcs*. GN235 also retained the ability to grow on vanillin and vanillic acid (Fig. 3).

Using transposon mutagenesis of GN235, we found a mutant (GN275) which was unable to grow on ferulic acid or vanillin as sole carbon sources. However, growth on vanillic acid was retained (Fig. 3). Identification of the gene disrupted by the transposon revealed *modA* (PP_3828), which encodes a periplasmic molybdate-binding protein, which is part of a molybdate ABC transporter. The whole operon including *modABC* (PP_3827 – PP_3832) was deleted markerlessly with the *upp* counterselection method resulting in strain GN276. The phenotype of this strain was the same as the transposon mutant (Fig. 3).

Bioconversion assays of strains GN23, GN235, and GN276

Resting cells of strains GN23 and GN235 were used for bioconversion assays. Ten millimolar of ferulic acid was

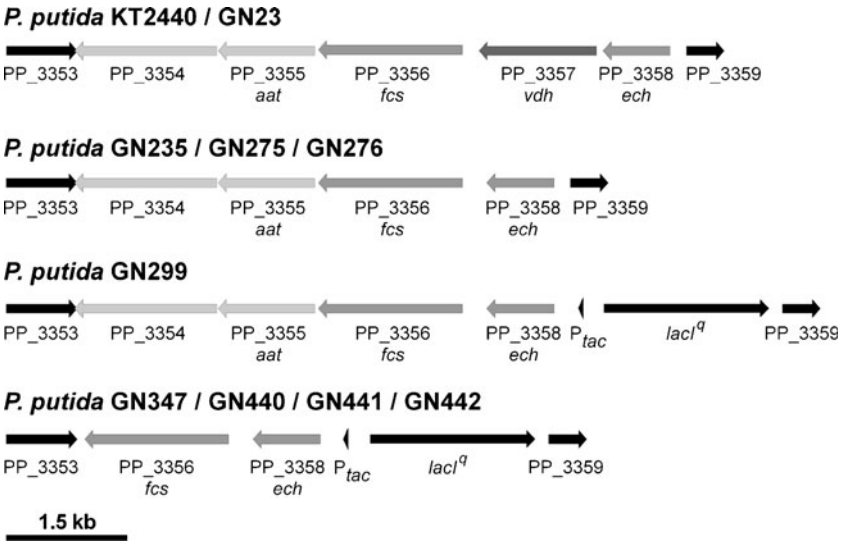


Fig. 2 Organization of the structural genes of the enoyl-CoA hydratase/aldolase (*ech*), feruloyl-CoA synthetase (*fcs*), and vanillin dehydrogenase (*vdh*), β-ketothiolase (*aat*) and acetyl-CoA dehydrogenase (PP_3354) in the *P. putida* mutant strains used in this study. The integration site of the *tac* promoter region including the *lac* operator (P_{tac}) and the gene for the *lac* repressor ($lacI^q$) is depicted

added to the resting cells and the concentrations of ferulic acid, vanillin, vanillyl alcohol and vanillic acid were measured by HPLC taking samples at regular intervals. The assay was stopped after 18 h conversion time. Both strains, GN23 and GN235, showed a rapid conversion of ferulic acid accompanied with a temporary accumulation of vanillic acid in the first 5 h (Fig. 4a, b). Furthermore, accumulation of vanillin, vanillyl alcohol, and vanillic acid could not be observed in either of them.

A bioconversion assay of ferulic acid with GN276 (Fig. 4c) showed a decreased conversion rate of ferulic acid. Whereas with GN23 all of the applied ferulic acid (10 mM) was converted after 18 h, 2.4 mM could still be measured using GN276. In contrast to GN23 and GN235, GN276 accumulated 4.8 mM vanillin after 5 h conversion time. Vanillin concentration slighlty increased to 5.2 mM after further 13 h conversion. At the end of the conversion (18 h), also vanillyl alcohol and vanillic acid were accumulated up to 1.5 and 0.3 mM, respectively. To improve the ferulic acid conversion rate, further steps were necessary.

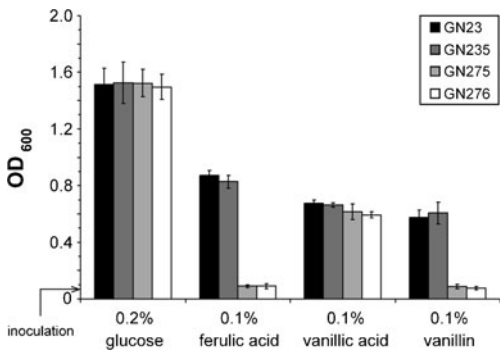


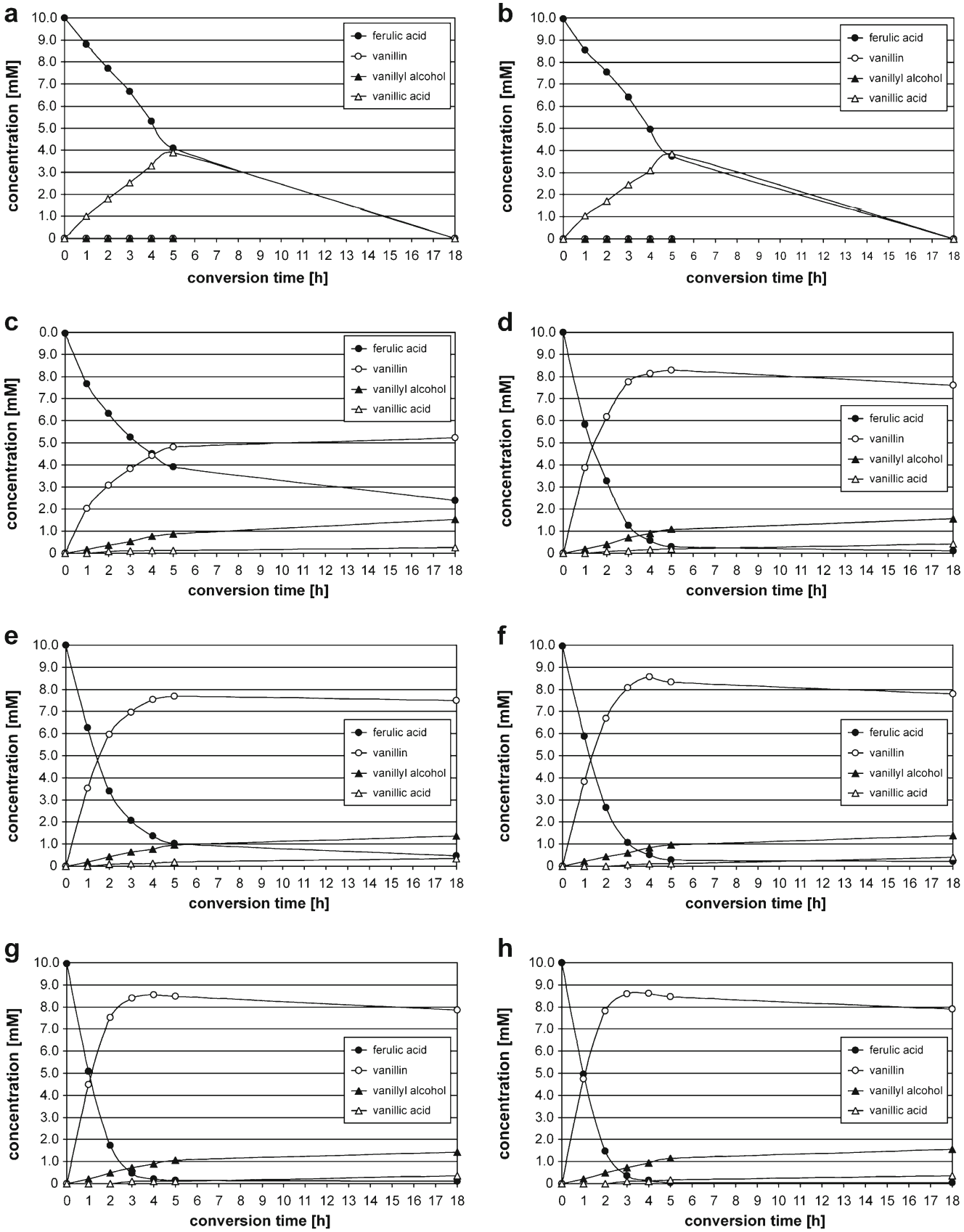
Fig. 3 Growth of *P. putida* mutant strains GN23, GN235, GN275, and GN276 in M9 minimal medium with different carbon sources. Strains were inoculated with 0.05 OD_{600} as indicated by an *arrow*. Growth was documented by measuring the OD_{600}. The OD_{600} after 24 h at 30 °C is presented to show the ability of the strains to grow on glucose, ferulic acid, vanillic acid, and vanillin, respectively, as sole carbon source

Increase of chromosomal *ech-fcs* expression leads to high conversion rates and high vanillin molar yields

Feruloyl-CoA-synthetase (encoded by *fcs*) and enoyl-CoA-hydratase/aldolase (encoded by *ech*) catalyze the conversion of ferulic acid to vanillin. We assumed that the conversion rate of ferulic acid should be directly proportional to the number of these two metabolic enzymes in the cell, if the required cofactors, ATP and CoA-SH, are available in excess or regenerated. Using the *upp* counterselection system, the strong *tac* promoter (P_{tac}) and $lacI^q$ were integrated immediately upstream of *ech* and *fcs* in the chromosome of GN276 in order to control the expression of these two genes (Fig. 2).

The resulting strain was designated GN299. After induction of *ech* and *fcs* expression with IPTG, bioconversion assays were conducted with this strain. After 5 h, nearly all of the 10 mM ferulic acid was converted to 1.1 mM vanillyl alcohol, 0.2 mM vanillic acid, and 8.3 mM of vanillin, corresponding to a molar yield of 83 % (Fig. 4d). After 18 h conversion, the vanillin concentration slightly decreased to 7.6 mM accompanied with an increase of vanillyl alcohol and vanillic acid to 1.6 and 0.4 mM, respectively.

Fig. 4 Bioconversion assays of ferulic acid to vanillin. Metabolic genes *ech* and *fcs* were induced for 6 h with 5 mM inducer before bioconversion of ferulic acid to vanillin was started with 5×10^9 resting cells ml^{-1} of *P. putida* strains **a** GN23; **b** GN235; **c** GN276; **d** GN299; **e** GN347; **f** GN440; **g** GN441; and **h** GN442. Concentrations of ferulic acid (*black circles*), vanillin (*white circles*), vanillyl alcohol (*black triangle*), and vanillic acid (*white triangle*) were measured by HPLC and plotted over the conversion time. The figure shows the mean values of at least three independently repeated assays. The standard deviation was less than 10 %


a
b
c
d
e
f
g
h
concentration [mM]
conversion time [h]
ferulic acid
vanillin
vanillyl alcohol
vanillic acid

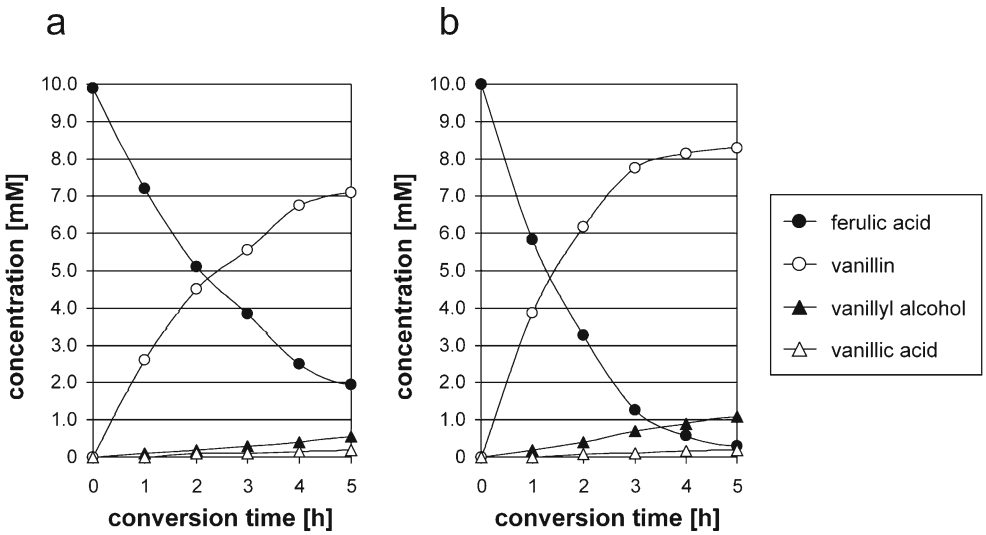


Fig. 5 Influence of the amount of inducer on the bioconversion of ferulic acid to vanillin. Cells of *P. putida* GN299 were induced for 6 h with **a** 1 mM IPGT and **b** 5 mM IPTG before bioconversion of ferulic acid to vanillin was started with 5×10^9 resting cells ml^{-1}. Concentrations of ferulic acid (*black circles*), vanillin (*white circles*), vanillyl alcohol (*black triangle*), and vanillic acid (*white triangle*) were measured by HPLC and plotted over the conversion time. The figure shows the mean values of at least three independently repeated assays. The standard deviation was less than 10 %

Optimization of the bioconversion assay revealed a treshold for vanillin

Resting cells of *P. putida* GN299 were used for bioconversion experiments. Several parameters were varied aiming a high and reproducible product yield combined with a high initial conversion rate.

First, we analyzed the influence of the inducer concentration (1 and 5 mM IPTG) and of the induction time (2, 4, and 6 h) for the expression of the metabolic enzymes needed for the bioconversion of ferulic acid to vanillin (Figs. 5 and 6a). We found that conversion of ferulic acid was much slower using less than 5 mM IPTG (Fig. 5). However, the vanillin yields after 18 h conversion time were similar (not shown). Regarding the influence of the induction time (Fig. 6a), the highest conversion rates and vanillin yields were found after 6 h induction of the metabolic enzymes.

Furthermore, the amount of resting cells was also varied (5, 10, and 20×10^9 cells ml^{-1}). The best results were aimed with the lowest concentration of 5×10^9 cells ml^{-1} (Fig. 6b). Higher cell concentrations led to raised levels of vanillyl alcohol and vanillic acid accompanied with a decrease in the vanillin molar yield after prolonged conversion (18 h).

In this study, we found that there is a threshold for vanillin production. Induced and resting cells were incubated with increasing amounts of ferulic acid in the bioconversion broth (10, 20, 30, and 40 mM). Using up to 30 mM ferulic acid, strains did not produce more than 13.5 mM vanillin (Fig. 7a).

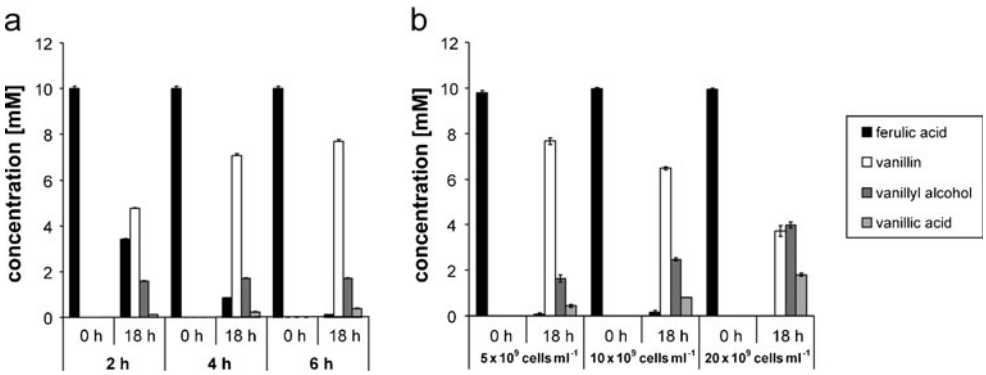


Fig. 6 Influence of the **a** induction time and **b** amount of resting cells of *P. putida* GN299 on the bioconversion of ferulic acid to vanillin. **a** Cells were induced for 2, 4, and 6 h with 5 mM IPTG before bioconversion of ferulic acid to vanillin was started with 5×10^9 resting cells ml^{-1}. **b** Cells were induced for 6 h with 5 mM IPTG before bioconversion of ferulic acid to vanillin was started with varying amounts of resting cells (5, 10, and 20×10^9 cells ml^{-1}). Concentrations of ferulic acid (*black bars*), vanillin (*white bars*), vanillyl alcohol (*dark gray bars*), and vanillic acid (*light gray bars*) were measured by HPLC and shown at the beginning (0 h) and at the end (18 h) of the bioconversion assay. The figure shows the mean values of at least three independently repeated assays. The standard deviation is represented by *error bars*

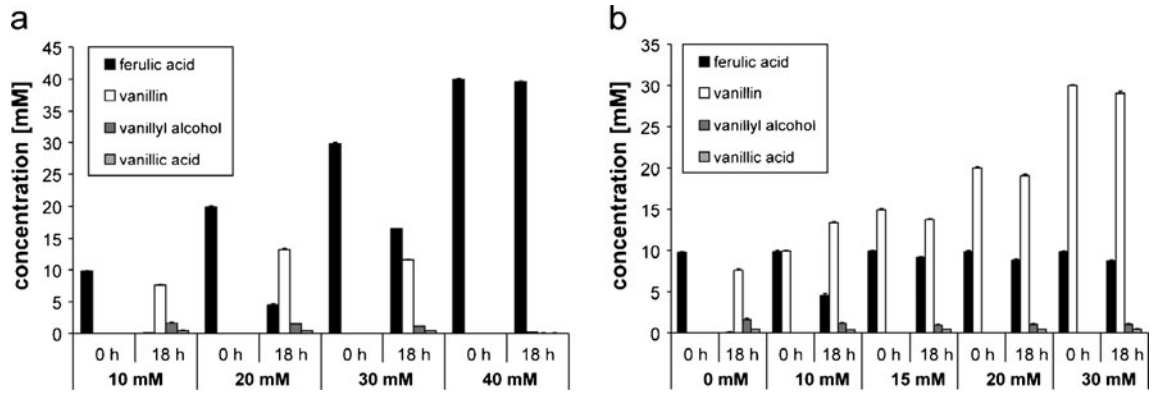


Fig. 7 Influence of **a** ferulic acid and **b** vanillin concentration on the bioconversion. Metabolic genes *ech* and *fcs* were induced for 6 h with 5 mM IPTG before bioconversion of ferulic acid to vanillin was started with 5×10^9 resting cells ml^{-1} of *P. putida* GN299. **a** Increasing concentrations of ferulic acid (10, 20, 30, and 40 mM) were used for the conversion to vanillin. **b** Increasing concentrations of vanillin (0, 10, 15, 20, and 30 mM) were added at the beginning of the bioconversion assay with 10 mM ferulic acid. Concentrations of ferulic acid (*black bars*), vanillin (*white bars*), vanillyl alcohol (*dark gray bars*), and vanillic acid (*light gray bars*) were measured by HPLC and shown at the beginning (0 h) and at the end (18 h) of the bioconversion assay. The figure shows the mean values of at least three independent assays. The standard deviation is represented by *error bars*

With 40 mM ferulic acid, no vanillin was produced at all. The best yields were achieved using 10 mM ferulic acid for the bioconversion assay. Growth kinetics of *P. putida* KT2440 mutant strains in buffered M9 minimal medium (pH 7.0) with glucose and increasing amounts of ferulic acid (0–50 mM) showed no influence of the ferulic acid concentration (Fig. 8a).

The vanillin threshold effect was confirmed by incubation of resting cells with 10 mM ferulic acid and additional increasing amounts of vanillin (10, 15, 20, and 30 mM). The cells incubated with additional 10 mM vanillin produced only further 3.2 mM vanillin after 18 h (Fig. 7b). On the other hand, higher amounts of vanillin resulted in a slight decrease of vanillin concentration and an increase of vanillyl alcohol and vanillic acid concentrations. Growth kinetics of *P. putida* KT2440 mutant strains in M9 minimal medium with glucose and increasing amounts of vanillin (0–25 mM) showed significant influence of the vanillin concentration (Fig. 8b). With up to 12.5 mM vanillin, the strains showed moderate growth. With over 15 mM vanillin, growth was strongly impaired.

Deletion of further genes and consequences on vanillin production and by-product formation

The bioconversion assays conducted with GN299 showed formation of vanillyl alcohol and vanillic acid which inevitably reduce the product yield. Therefore, the effect of the inactivation of several genes potentially involved in the ferulic acid metabolism was analyzed. The genes chosen for this analytical approach were PP_3354 (acyl-CoA dehydrogenase) and PP_3355 (β-ketothiolase), PP_2680 and PP_0545 (aldehyde dehydrogenases), and PP_1948 (benzaldehyde dehydrogenase).

First, a second pathway from ferulic to vanillic acid (Fig. 1) proposed by Overhage et al. (1999b) was interrupted in strain GN299 by combined deletion of PP_3354 and PP_3355

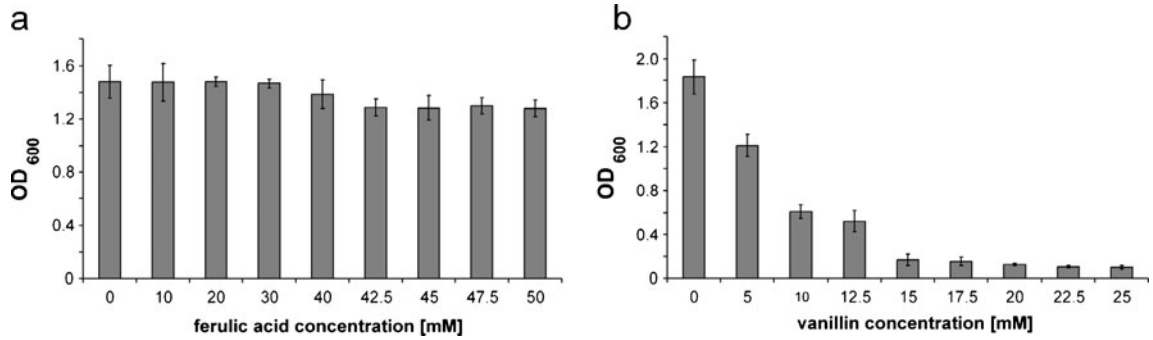


Fig. 8 Tolerance of *P. putida* mutant strain GN299 towards different **a** ferulic acid and **b** vanillin concentrations in M9 minimal medium. After inoculation with 0.1 OD_{600} in M9 minimal medium with 0.4 % glucose and increasing concentrations of **a** ferulic acid and **b** vanillin, growth was documented by measuring the OD_{600}. The OD_{600} after 24 h at 30 °C is presented to show the tolerance of GN299 towards different concentrations of ferulic acid and vanillin, respectively. The figure shows the mean values of at least three independent assays. The standard deviation is represented by *error bars*

coding for an acyl-CoA dehydrogenase and a β-ketothiolase (*aat*) as depicted in Fig. 2. The resulting strain GN347 was used for bioconversion assays (Fig. 4e). After 5 h, 9 mM ferulic acid was converted to 7.7 mM vanillin, 1 mM vanillyl alcohol, and 0.2 mM vanillic acid. After 18 h, further 0.5 mM ferulic acid was converted. The vanillin concentration decreased to 7.5 mM, whereas vanillyl alcohol and vanillic acid slightly increased to 1.4 and 0.3 mM, respectively. Compared to GN299, the conversion rate and vanillin yield within the first 5 h decreased.

Since deletion of *vdh* could not prevent degradation of vanillin to vanillic acid, other aldehyde dehydrogenases may catalyze this reaction. From a proteomics approach, it could be shown that two aldehyde dehydrogenases (encoded by PP_2680 and PP_0545) and the benzaldehyde dehydrogenase (PP_1948) were upregulated in *P. putida* KT2440 growing on vanillin (Simon et al., unpublished, manuscript in preparation). Sequential inactivation of PP_2680 and PP_0545 in GN299 by markerless deletion resulted in strains GN440 and GN441, respectively. The benzaldehyde dehydrogenase may also accept vanillin as a substrate, since it is a derivative of benzaldehyde. Therefore, the corresponding gene (PP_1948) was deleted in GN441, resulting in strain GN442. The mutant strains GN440, GN441, and GN442 were used in bioconversion assays (Fig. 4f–h). The strains showed very similar results. After 4 h, about 9.5 mM ferulic acid was converted to 8.6 mM vanillin with resting cells of GN440 and GN441. The measured vanillyl alcohol and vanillic acid concentrations were about 0.8 and 0.1 mM, respectively. Strain GN442 showed the same results, however, already after 3 h conversion. After 18 h conversion, nearly all of the ferulic acid was converted with all three strains. Again, vanillin concentration decreased to 7.9 mM, whereas vanillyl alcohol and vanillic acid increased to about 1.5 and 0.4 mM, respectively.

Discussion

In contrast to *P. fluorescens* strains AN103 and BF13 (Martinez-Cuesta et al. 2005; Di Gioia et al. 2010), our findings with *P. putida* GN235 confirm that simple inactivation of *vdh* is not sufficient to prevent vanillin degradation. This was reported previously with a *vdh* knockout mutant of *Pseudomonas* sp. HR199 strain and a *Pseudomonas* KT2440*vdh*ΩKm mutant (Overhage et al. 1999a; Plaggenborg et al. 2003). KT2440*vdh*ΩKm and GN235 were still able to grow on vanillin as sole carbon source. The main difference, however, was that *P. putida* GN235 was also able to grow on ferulic acid, due to a functional expression of the adjacent genes of *vdh*, namely *ech* and *fcs*. The clean deletion of *vdh* sustained expression of *ech* and *fcs*, which was most probably not the case in the KT2440*vdh*ΩKm mutant. A random transposon mutagenesis conducted with GN235 revealed a mutant with a transposon in the gene locus of *modA*, which encodes for a periplasmic molybdate-binding protein. Molybdate ions are known to play a role as cofactors in oxidoreductases in *Pseudomonas* species (Koenig and Andreesen 1990; Blaschke et al. 1991; Frunzke et al. 1993). Since the Δ*modABC* strain GN276 was not able to grow on vanillin as sole carbon source and we also did not find mutants with a disrupted gene encoding for a specific oxidoreductase, it can be assumed that there are more than one unknown molybdate depending oxidoreductases, which may accept vanillin as a substrate complementing the *vdh* inactivation. The inhibition of the molybdate uptake could limit the activities of these enzymes in such a way that vanillin is not further oxidized to promote growth.

Since ferulic acid was not completely converted with GN276, further improvements were necessary. A concurrent expression of the structural genes *ech* and *fcs* on a low-copy plasmid in a *vdh* negative *P. fluorescens* strain led to a vanillin molar yield of 63 % within 5 h using resting cells from shaken flask experiments and up to 84 % within 24 h using resting cells from a stirred tank reactor (Di Gioia et al. 2010). To circumvent possible problems of plasmid instabilities and usage of antibiotics, the strong *tac* promoter was introduced into the chromosome of *P. putida* GN276 to control expression of *ech* and *fcs* (GN299). This improved the vanillin molar yield up to 83 % within just 5 h. We assume that raising the expression rate of *ech* and *fcs* through induction with IPTG led to higher concentrations of the encoded metabolic enzymes than using the original promoter system. In contrast to ferulic acid, the inducer IPTG gets not metabolized and the expression rates can stay on a high level. Lowering the amount of inducer led to a decrease in product yield and productivity, which can be explained by lower enzyme concentrations. We also checked the effect of induction time, showing that less than 6 h resulted in lower product yields, probably due to lower enzyme levels. Longer induction times were also checked, but did not improve the product yields (data not shown). Raising the cell concentration in the assay led to higher levels of the by-products vanillic acid and vanillyl alcohol, and did not accelerate the conversion time. We assume that higher cell densitites are accompanied with higher levels of reduction equivalents which in turn may favor the formation of vanillyl alcohol.

Further improvements of the conversion process showed that raising the concentration of ferulic acid in the bioconversion broth results in a reduction of the vanillin molar yield, if concentrations higher than 10 mM of ferulic acid are used. A ferulic acid concentration of 40 mM even inhibited any conversion to vanillin. A toxic effect of ferulic acid, however, could be excluded, as growth experiments with increasing amounts of ferulic acid with up to 50 mM have shown.

On the other hand, *P. putida* GN299 showed a vanillin threshold of about 13.5 mM in the bioconversion assays.

Raising the vanillin concentration above this threshold led to formation of more vanillyl alcohol and vanillic acid, and inhibited conversion of ferulic acid. Such a product inhibition was also observed with recombinant *E. coli* strains converting ferulic acid to vanillin (Overhage et al. 2003). The toxic character of vanillin was confirmed with growth experiments of *P. putida* GN299 in the presence of increasing vanillin concentrations, where only up to 12.5 mM vanillin was tolerated. In contrast to *P. fluorescens* BF13, however, which showed a 98 % reduction of the molar yield by increasing the ferulic acid concentration from 5 to 12.5 mM (Di Gioia et al. 2010), *P. putida* did not show such sensible reductions. Indeed, resting cells of *P. putida* GN442 could be reused after conversion for 18 h. Distracting vanillin by resuspending cells in new buffer with 10 mM ferulic acid and further incubation at 30 °C for 18 h resulted in production of 5 mM vanillin (4.5 mM ferulic acid, 0.5 mM vanillyl alcohol, 0 mM vanillic acid). Therefore, immediate distraction of the toxic product vanillin by adsorbent resins would allow *P. putida* cells to convert more ferulic acid as it could be shown previously for other systems (Yoon et al. 2007; Hua et al. 2007; Lee et al. 2009).

Inactivation of the alternative pathway from ferulic to vanillic acid proposed by Overhage et al. (1999b) by deletion of PP_3355 (*aat*) and PP_3354 in GN299 had no positive effect on formation of the unwanted formation of the by-products vanillyl alcohol and vanillic acid. It was observed that the conversion rate even decreased. A possible explanation for this behavior could be a shorter half-life of the mRNA provoked by the deletion of the two genes and therefore a diminished level of the metabolic enzymes. However, inactivation of the upregulated aldehyde dehydrogenases, encoded by PP_2680 and PP_0545, and the benzaldehyde dehydrogenase (PP_1948) in GN299, led to higher initial conversion rates and high molar yields. The final titer and productivity of vanillin of 8.6 mM and 2.87 mmol l^{-1} h^{-1} with this strain (GN442) outnumber the highest levels to date of 8.41 mM and 0.35 mmol l^{-1} h^{-1}, which have been achieved with a *Pseudomonas* strain concerning the bioconversion of ferulic acid to vanillin (Di Gioia et al. 2010). However, the deletions had no significant effect on formation of the by-products compared to GN299. In stirred tank reactor experiments, it could previously be shown that raising the dissolved oxygen concentrations did not result in formation of more vanillic acid excluding a chemical oxidation process (Di Gioia et al. 2010). It was proposed that other broad substrate specificity dehydrogenases may act in *Pseudomonas* strains that have to be determined yet (Overhage et al. 1999b).

All our bioconversion experiments showed that prolonged bioconversion times up to 18 h reduced the vanillin molar yield due to formation of the by-products. Therefore, a fast and complete as possible conversion of ferulic acid to vanillin is desirable in the first few hours. The reduction of vanillin to vanillyl alcohol seems to represent a detoxification mechanism like it was also observed with recombinant *E. coli* cells converting ferulic acid to vanillin (Overhage et al. 2003). Another approach to reduce the formation of vanillyl alcohol was to lower the amount of $NADH_2$ by deletion of the genes PP_4011 and PP_4012, encoding the isocitrate dehydrogenase, like it was proposed for recombinant *E. coli* (Lee et al. 2009). Unfortunately, deletion by the *upp* counterselection system as well as simple gene disruptions failed.

Further investigations will focus on finding the dehydrogenases responsible for formation of vanillyl alcohol. Another aspect of the established and promising bioconversion system with *P. putida* GN442 is the use of adsorbent resins to reduce the toxicity of the product vanillin and upscaling, in order to prove the versatility in industrial processes. It would also be interesting to know if resting cells of *P. putida* strains grown in a stirred tank reactor do not show any formation of vanillyl alcohol, like it was stated for the *P. fluorescens* BF13 system (Di Gioia et al. 2010).

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