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Improved \(p\)-hydroxybenzoate production by engineered \textit{Pseudomonas putida} S12 by using a mixed-substrate feeding strategy

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Abstract The key precursors for \(p\)-hydroxybenzoate production by engineered \textit{Pseudomonas putida} S12 are phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), for which the pentose phosphate (PP) pathway is an important source. Since PP pathway fluxes are typically low in pseudomonads, E4P and PEP availability is a likely bottleneck for aromatics production which may be alleviated by stimulating PP pathway fluxes via co-feeding of pentoses in addition to glucose or glycerol. As \textit{P. putida} S12 lacks the natural ability to utilize xylose, the xylose isomerase pathway from \textit{E. coli} was introduced into the \(p\)-hydroxybenzoate producing strain \textit{P. putida} S12\(\text{palB2}\). The initially inefficient xylose utilization was improved by evolutionary selection after which the \(p\)-hydroxybenzoate production was evaluated. Even without xylose-co-feeding, \(p\)-hydroxybenzoate production was improved in the evolved xylose-utilizing strain, which may indicate an intrinsically elevated PP pathway activity. Xylose co-feeding further improved the \(p\)-hydroxybenzoate yield when co-fed with either glucose or glycerol, up to 16.3 Cmol\% (0.1 g \(p\)-hydroxybenzoate/g substrate). The yield improvements were most pronounced with glycerol, which probably related to the availability of the PEP precursor glyceraldehyde-3-phosphate (GAP). Thus, it was demonstrated that the production of aromatics such as \(p\)-hydroxybenzoate can be improved by co-feeding different carbon sources via different and partially artificial pathways. Moreover, this approach opens new perspectives for the efficient production of (fine) chemicals from renewable feedstocks such as lignocellulose that typically has a high content of both glucose and xylose and (crude) glycerol.

Keywords \textit{Pseudomonas putida} S12 · Aromatics · Mixed substrate feeding · Glucose · Xylose

Introduction

\textit{Pseudomonas putida} S12 is a solvent-tolerant bacterium that has been developed as a platform host for the production of
a range of substituted aromatic compounds such as phenol, t-cinnamate, p-coumarate, p-hydroxybenzoate, and p-hydroxystyrene (Nijkamp et al. 2005; Nijkamp et al. 2007; Verhoef et al. 2007; Verhoef et al. 2009; Wierckx et al. 2005). Its solvent tolerance properties enable *P. putida* S12 to produce these toxic hydrophobic compounds to high titres without provoking harmful effects (de Bont 1998). Furthermore, in situ product extraction can be applied in fermentations by adding a second phase of a water-immiscible solvent, preventing the accumulation of product to concentrations that are inhibitory even to solvent-tolerant microorganisms (Heipieper et al. 2007; Verhoef et al. 2009).

The production of aromatic compounds by engineered *P. putida* S12 is based on the conversion of endogenously formed tyrosine or phenylalanine. The key precursors of these aromatic amino acids are phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) (Fig. 1). PEP is produced in the lower glycolysis from glyceraldehyde-3-phosphate (GAP). GAP is formed from glucose, either via the Entner-Doudoroff pathway or via the pentose phosphate (PP) pathway, whereas E4P is derived exclusively from the PP pathway. In view of the typically low activity of the PP pathway in *P. putida* (del Castillo et al. 2007; Fuhrer et al. 2005; Wierckx et al. 2009), the availability of E4P and PEP may present a bottleneck for efficient aromatics production. Increasing the availability of E4P and PEP was therefore expected to enhance the production of aromatic compounds by engineered *P. putida* S12, as previously demonstrated for the pre-aromatic compounds chorismate and shikimate in *Escherichia coli* (Martinez et al. 2008).

The availability of PEP and E4P may be improved by stimulating PP pathway fluxes through pentose (co-) feeding, as was demonstrated previously in *E. coli* (Gonzalez et al. 2002). Unfortunately, this strategy cannot be applied to *P. putida* S12 as this strain lacks the natural ability to utilize pentoses. However, in previous work, we successfully introduced xylose utilization, via the xylose isomerase and PP pathway, into wild-type *P. putida* S12 (Meijnen et al. 2008). In the present study, a similar approach was employed to introduce xylose catabolism into *P. putida* S12palB2. This *P. putida* S12-derived strain produces p-hydroxybenzoate, which was selected as a model value-added aromatic compound derived from the aromatic amino acid biosynthesis pathway (Verhoef et al. 2010). The effect of xylose co-feeding on p-hydroxybenzoate production was assessed using glucose as the primary

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**Fig. 1** Schematic representation of the biosynthetic pathways for p-hydroxybenzoate production from glycerol, glucose, and xylose. The scheme shows only the relevant routes. Heterologous genes are indicated in *italics* and *underlined*. Xylose isomerase (*xylA*); xylulokinase (*xylB*); phenylalanine/tyrosine ammonia lyase (*pal*/*tal*); Glucose-6-phosphate (*G6P*); fructose-6-phosphate (*F6P*); fructose-1,6-bisphosphate (*F1,6BP*); triose-3-phosphate (*T3P*); phosphoenolpyruvate (*PEP*); pyruvate (*PYR*); glycerol-3-phosphate (*Gly3P*); ribulose-5-phosphate (*Ru5P*); xylulose-5-phosphate (*Xu5P*); ribose-5-phosphate (*R5P*); glyceraldehyde-3-phosphate (*GAP*); sedoheptulose-7-phosphate (*S7P*); erythrose-4-phosphate (*E4P*); 3-deoxy-D-arabino-heptulosonate-7-phosphate (*DAHP*); chorismate (*CHO*); phenylalanine (*PHE*); cinnamate (*CIN*); tyrosine (*TYR*); p-coumarate (*COUM*); 4-hydroxyphenylpyruvate degradation pathway (*HD pathway*); protocatechuate degradation pathway (*PD Pathway*)
carbon source, mimicking lignocellulosic hydrolysate that typically contains high levels of both glucose and xylose. Alternatively, glycerol was employed as primary carbon source, being a good source for PEP as well as being a model for raw glycerol waste from biodiesel production.

**Materials and methods**

**Bacterial strains, plasmids, and culture conditions**

The strains and plasmids used in this study are listed in Table 1. The media used were Luria broth (LB) (Sambrook et al. 1982) and a phosphate-buffered minimal medium (MM; Verhoef et al. 2010). In minimal media, 12 mM of xylose (MMX), 10 mM of glucose (MMG), or 20 mM of glycerol (MMGly) was used as sole carbon source, unless stated otherwise. Antibiotics were added as required to the media to the following final concentrations: gentamicin, 25 μg ml⁻¹ (MM) or 40 μg ml⁻¹ (LB); tetracycline, 40 μg ml⁻¹ (P. putida S12 in MM) or 60 μg ml⁻¹ (P. putida S12 in LB). In view of the photosensitivity of tetracycline, amber bottles and light tight incubators were employed for culturing. The expression of the photosensitivity of tetracycline, amber bottles and light tight incubators were employed for culturing. The expression of the photosensitivity of tetracycline, amber bottles and light tight incubators were employed for culturing. The expression of the photosensitivity of tetracycline, amber bottles and light tight incubators were employed for culturing. The expression of the photosensitivity of tetracycline, amber bottles and light tight incubators were employed for culturing. The expression of the photosensitivity of tetracycline, amber bottles and light tight incubators were employed for culturing.

**Table 1** Strains and plasmids used in this study

| Strain or plasmid | Characteristics | Source |
|-------------------|-----------------|--------|
| **Strains**       |                 |        |
| *P. putida* S12   | Wild type, ATCC 700801 | (Hartmans et al. 1990) |
| *P. putida* S12B2 | *pohB* and *hpd* knockout strain with an enhanced flux towards tyrosine | (Verhoef et al. 2010) |
| *P. putida* S12palB2 | *P. putida* S12B2 containing plasmid pJT'Tpal | (Verhoef et al. 2010) |
| *P. putida* S12B6 | *gcd* knockout strain derived from *P. putida* S12B2 | This study |
| *P. putida* S12palB6 | *P. putida* S12B6 containing plasmid pBT'Tpal | This study |
| *P. putida* S12xylB6 | *P. putida* S12B6 containing plasmid pJNTxylAB_FGH | This study |
| *P. putida* S12xylB7 | *P. putida* S12xylB6 evolved to efficient xylose user | This study |
| *Escherichia coli* DH5α | *supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1* | Invitrogen |
| **Plasmids**      |                 |        |
| pJT'Tmcs          | Ap⁺, Gm⁺, basic expression vector for Ptac and tac RBS-controlled expression | (Verhoef et al. 2010) |
| pJNTmcs(t)        | Ap⁺, Gm⁺, basic expression vector containing the salicylate-inducible promoter nagAa and tac RBS | (Meijnen et al. 2008) |
| pJNTxylAB_FGH     | pJNTmcs(t) containing the *xylAB_FGH* genes from *E. coli* DH5α | (Meijnen et al. 2008) |
| pBT'Tmcs          | Tc⁺, basic expression vector for Ptac and tac RBS controlled expression | This study |
| pBT'Tpal          | pBT'Tmcs containing the *pal* gene from *R. toruloides* | This study |
| pJO200SK          | P15A ori sacB RP4 Gmr(BluescriptSK); suicide vector | (Quandt and Hynes 1993) |
| pJOgcd::tetA_loxP | Gm⁺ Tc⁺, pJO200SK containing a *loxP*-tetA-locP interrupted copy of the *gcd* gene | This study |

*Ap⁺, Gm⁺ and Tc⁺, ampicillin, gentamicin, and tetracycline resistance, respectively
Upon reaching steady state at \( D = 0.1 \text{ h}^{-1} \), the cultures were sampled again. For glucose–xylose mixtures, the \( D \) was set at 0.1 \( \text{h}^{-1} \). The cultures were considered to be at steady state when no significant changes were measured in cell density, stirring speed, and \( p \)-hydroxybenzoate concentration after at least five volume changes at the corresponding \( D \).

Analytical methods

Optical densities were measured at 600 nm (OD_{600}) using an Ultrospec Cell Density Meter (GE Healthcare). An optical density of 1.0 corresponds to a cell dry weight (CDW) of 0.49 g l\(^{-1}\). \( p \)-Hydroxybenzoate was analyzed by HPLC (Agilent 1100 system) using a Zorbax 3.5 \( \mu \text{m SB-C18 column} (4.6 \times 50 \text{ mm}) and a diode-array detector set at 254 nm. As the eluent, a linear gradient of acetonitrile in KH\(_2\)PO\(_4\)-buffer (50 mM, pH 2, 1% acetonitrile) was used, increasing from 0% to 25% in 4.9 min at a flow of 1.5 ml min\(^{-1}\). Glucose, xylose, and glycerol were analyzed with a Dionex ICS3000 system as described previously (Meijnen et al. 2008; Verhoef et al. 2010).

DNA techniques

Genomic DNA was isolated using the DNeasy Blood & Tissue kit (QIAGEN). PCR reactions were performed with Accuprime \( Pfx \) polymerase (Invitrogen) according to the manufacturer’s instructions. Plasmid DNA was isolated with the QIAprep spin miniprep kit (QIAGEN). DNA concentrations were measured with the ND-1000 spectrophotometer (Nanodrop). Agarose-trapped DNA fragments were isolated with the QIAEXII gel extraction kit (QIAGEN). Plasmid DNA was introduced into electrocompetent cells using a Gene Pulser electroporation device (BioRad). DNA sequencing reactions were performed by Eurofins MWG Operon (Ebersberg, Germany).

Construction of expression plasmids

For constructing plasmid pBTTmcs (Te\(^{c} \)) the \( tac \) expression cassette and chloramphenicol (Cm) marker of pJTTmcs (Verhoef et al. 2010) were amplified by PCR using primers 1 and 2 (Table 2). The resulting PCR product was digested with \( Kpn2I \) and \( XmaII \) (restriction sites present in the amplified fragment) and consequently ligated in a \( Kpn2I- XbaI \) compatible with \( XmaII \) digested pBBR1mcs vector, yielding pBTTmcs (Cm\(^{c} \)). The Cm marker was replaced by a tetracycline (Tc) marker, which was obtained by PCR using primers 3 and 4 (Table 2) on vector pTO1 (Kieboom and de Bont 2001) and cloned into the \( PsaI \) and \( NcoI \) restriction sites of pBTTmcs (Cm\(^{c} \)), yielding pBTTmcs (Te\(^{c} \)).

For constructing pBTTpal, the \( pal \) gene from pJTTpal (Verhoef et al. 2007) was obtained as a \( Kpn1-NotI \) fragment and purified from agarose gel. The purified fragment was ligated into \( Kpn1-NotI \)-digested pBTTmcs, yielding pBTTpal.

Expression vector pJNTxylAB\(_{FGH} \) was constructed by cloning \( xylA/B \) and \( xylFGH \) in vector pJNTmcs (Meijnen et al. 2008). The genes were amplified by PCR using genomic DNA from \( E. \ coli \) DH5\(^{a} \) as the template and oligonucleotide primers 5–8 (Table 2). The resulting fragments were ligated into vector pJNTmcs using the restriction sites \( Kpn1 \) and \( NotI \) for \( xylAB \), and \( Nhel \) and \( SfiI \) for \( xylFGH \). The resulting plasmid was designated pJNTxylAB\(_{FGH} \).

Targeted gene disruption

The \( gcd \) knockout mutant of \( P. \ putida \) S12B2 (Verhoef et al. 2010) was constructed in analogy to the \( gcd \) knockout mutant of wild-type \( P. \ putida \) S12 (Meijnen et al. 2008). The gene replacement plasmid for the \( gcd \) gene, pJQgcd::tetAloxP was constructed from the suicide vector pJQ200SK (Quandt and Hynes 1993) based on the pJQgcd::Kana vector (Meijnen et al. 2008). The \( loxP-\text{kanaR}-\text{loxP} \) fragment, encoding kanamycin resistance in vector pJQgcd::Kana was replaced by the \( loxP-\text{tetA}-\text{loxP} \) fragment (Sauer and Henderson 1988; Sterberg and Hamilton 1981), coding for tetracycline resistance, using \( XbaI \).

Results

Construction of a xylose-utilizing \( p \)-hydroxybenzoate-producing strain

In order to establish \( p \)-hydroxybenzoate production from xylose, the optimized base strain for \( p \)-hydroxybenzoate production, \( P. \ putida \) S12B2 (Table 1; (Verhoef et al. 2010)), was engineered for xylose utilization. First, the \( gcd \) gene encoding glucose dehydrogenase was disrupted in order to eliminate xylose oxidation, which makes xylose effectively unavailable for the xylose isomerase pathway (Meijnen et al. 2008). Subsequently, the \( xylAB_{FGH} \) genes from \( E. \ coli \) DH5\(^{a} \) (encoding xylose isomerase, xylulokinase, and a high-affinity xylose transporter) were introduced. The resulting strain, \( P. \ putida \) S12xylB6, showed very slow growth on minimal medium with xylose as the sole carbon source, requiring 16 days to reach a cell dry weight (CDW) of 0.69 g l\(^{-1}\). This result was in agreement with previous findings, and evolutionary selection was performed to improve xylose utilization as described previously (Meijnen et al. 2008). After four transfers, \( P. \ putida \) S12xylB7 was obtained that exhibited a maximum substrate yield \( (Y_{ss}) \) of 51 Cmol%.

The phenylalanine/tyrosine ammonia lyase (Pal/Tal) expression plasmid pBTTpal was introduced into \( P. \ putida \)
S12xylB7 to establish \( p \)-hydroxybenzoate production, yielding strain \( P.\ putida \) S12pal_xylB7. Remarkably, upon introduction of \( pBT'Tpal \), the maximum growth rate on xylose was reduced by a factor 3 compared to the plasmid-free strain, whereas the growth rate on glucose was not affected (results not shown). Still, \( p \)-hydroxybenzoate was efficiently produced from xylose in batch cultivations, at a product-to-substrate yield (\( Y_{ps} \)) of 12.4 Cmol% (Table 3). Another remarkable observation was the apparently improved \( p \)-hydroxybenzoate yield after evolutionary selection. Compared to the non-evolved strain \( P.\ putida \) S12palB6, the product-to-substrate yield of strain S12xyl_palB7 on glucose had increased from 14.2 to 17.4 Cmol%. On glycerol, the product-to-substrate yield improved from 15.4 to 19.3 Cmol% (data not shown, respectively, Table 3).

Mixed-feed chemostats

In previous work on the engineered xylose-utilizing \( P.\ putida \) strain S12xylAB2, a diauxic shift was observed in batch cultivations on mixtures of glucose and xylose, glucose being the preferred carbon source (Meijnen et al. 2008). Obviously, the occurrence of diauxy would invalidate the concept of co-feeding two substrates simultaneously to different pathways, in order to improve the availability of two key precursors. Therefore, a chemostat cultivation setup was selected for the mixed-substrate experiments, with glucose or glycerol as the limiting nutrient. Varying amounts of xylose were used (0% to 75% of 60 mM total carbon) to replace the primary carbon source.

Although the primary carbon source was completely metabolized as expected, residual xylose was observed in the chemostat effluent. The extent to which xylose was utilized was furthermore very different for glucose and glycerol (Fig. 2). On glycerol–xylose mixtures, the amount of xylose consumed correlated well with the amount of xylose in the feed. The residual xylose observed was in the same range as in chemostat cultivations with xylose as the sole carbon source (approximately 0.27 mM). However, with glucose as the primary carbon source, the residual xylose concentrations were much higher and, furthermore, increased more than proportionally to the relative xylose concentration in the feed. Clearly, the capacity to transport and/or utilize xylose was dependent on the type of primary carbon source as well as on the relative amount of the primary carbon source in the feed.

Production of \( p \)-hydroxybenzoate from mixtures of glucose and xylose

Chemostat cultivations with \( P.\ putida \) S12pal_xylB7 on glucose as single carbon source showed a product-to-substrate yield of 4.9 Cmol%, with a specific production
Production of p-hydroxybenzoate from mixtures of glycerol and xylose

In chemostats with glycerol as the sole carbon source, *P. putida S12pal* _xyLB7_ produced p-hydroxybenzoate at a yield of 8.5 Cmol%. Notably, this represents a 1.7-fold improvement over the product yield observed in glycerol-grown cultures. Also in shake-flasks, an improved p-hydroxybenzoate yield on glycerol was observed (Table 3), although not as pronounced as in the chemostat cultivations. In addition to the product-to-substrate yield, also the biomass-to-substrate yield was improved 1.23-fold on glycerol (Fig. 3c). Because of the improved biomass yield, the effect of the primary substrate on the specific p-hydroxybenzoate production rate $q_p$ was limited: the $q_p$ on glycerol (8.57 μmol C (g CDW)$^{-1}$ h$^{-1}$) was only slightly higher than on glucose (7.96 μmol C (g CDW)$^{-1}$ h$^{-1}$).

The intrinsically improved p-hydroxybenzoate production from glycerol was further enhanced by xylose co-feeding. The product-to-substrate yield increased by a factor 1.9 to a maximum of 16.3 Cmol% (Fig. 3d). The $q_p$ improved along with $Y_{PS}$, to a maximum value of 18 μmol C (g CDW)$^{-1}$ h$^{-1}$. As observed for the glucose-grown chemostats, the relative amount of xylose in the feed did not affect the efficiency of p-hydroxybenzoate production from glycerol–xylose mixtures (Fig. 3d).

Production of p-hydroxybenzoate from xylose as single carbon source

In order to verify that the improved p-hydroxybenzoate production was a result of the simultaneous utilization of xylose and glucose or glycerol, and not merely of xylose utilization alone, chemostat experiments were performed with *P. putida S12pal* _xyLB7_ on xylose as single carbon source. In agreement with the reduced growth rate observed in xylose-grown batch cultures after introducing the *pal/tal* expression plasmid, *P. putida S12pal* _xyLB7_ washed out above a dilution rate of 0.05 h$^{-1}$. Therefore, chemostats on xylose were operated at a dilution rate of 0.05 h$^{-1}$. Also chemostats with a mixed glycerol–xylose feed, or glycerol as the sole carbon source, were operated at this dilution rate, as control for growth-rate effects on p-hydroxybenzoate production.

Biomass and product yields were not significantly different in chemostats operated at $D=0.05$ h$^{-1}$ and $D=0.1$ h$^{-1}$ (Fig. 3e–f), whereas the specific production and substrate uptake rates were proportional to the dilution rate as expected (Fig. 3e–f). On xylose as a single carbon source, the product-to-substrate yield (5.6 Cmol%) was 1.5-fold lower than on glycerol alone (Table 3), which result is in agreement with the product yields observed in shake flask cultures (Table 3). This demonstrates that the improved p-hydroxybenzoate production is indeed a result from co-feeding two carbon sources. Furthermore, as the product yield on mixtures of xylose and glycerol or glucose consistently exceeded the product yield on either individual carbon source (Table 3), it was concluded that co-feeding xylose to glucose or glycerol has a beneficial effect over feeding individual carbon sources for p-hydroxybenzoate production.

Discussion

A mixed-substrate feeding strategy was devised to improve aromatics production by engineered *P. putida* S12. The approach was based on the assumption that the precursors

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**Fig. 2** Relative xylose uptake as a function of the xylose fraction in the feed. The *solid line* represents the theoretical maximum uptake of xylose; the *dotted lines* represent the actual uptake of xylose with glycerol as co-substrate (triangles) or glucose as co-substrate (circles). Data are the average from two independent cultivations; *error bars* represent the maximum deviation from the mean.
E4P and PEP were limiting factors for aromatic biosynthesis and that their availability could be improved by stimulating the PP pathway fluxes through pentose co-feeding. A \(p\)-hydroxybenzoate producing strain, \(P.\ putida\) S12palB2, was selected as an aromatics-producing model system. As this strain does not have the natural ability to utilize pentoses, a xylose isomerase pathway was introduced, and the initially low growth rate on xylose was improved via an evolutionary selection procedure. Surprisingly, tenfold less transfers were required to achieve growth characteristics similar to those of the previously evolved xylose-utilizing strain \(P.\ putida\) S12xylAB2 (Meijnen et al. 2008). In part, this can be attributed to the targeted disruption of \(gcd\), since more than ten transfers had been required for strain S12xylAB2 to acquire a \(gcd\) negative phenotype. In addition to the xylose utilization efficiency, also the \(p\)-hydroxybenzoate yield was improved after the evolutionary selection. This phenomenon may be explained by the increased PP pathway activity associated with the improved xylose utilization phenotype, leading to an intrinsically improved E4P and PEP availability, independently from xylose co-feeding.

As anticipated, xylose co-feeding considerably improved the \(p\)-hydroxybenzoate yield. The increased product yield was observed with both glucose and glycerol as the primary substrate and was shown not to be caused by xylose consumption per se. Remarkably, both the product and biomass yield on glycerol were consistently higher com-

\[a\] glucose and xylose mixtures at a \(D\) of 0.1 h\(^{-1}\)

\[b\] glycerol and xylose mixtures at a \(D\) of 0.1 h\(^{-1}\)

\[c\] glycerol and xylose mixtures at a \(D\) of 0.05 h\(^{-1}\)

...
pared to glucose, either with or without xylose co-feeding. This may be attributed to regulatory effects (e.g., carbon catabolite repression) but could also indicate that PEP availability is more critical for efficient \( p \)-hydroxybenzoate production than E4P availability. If it is assumed that pyruvate dikinase (PEP synthase) is active only under gluconeogenic conditions (Sauer and Eikmanns 2005), twice the amount of GAP (and, thus, PEP) can be obtained from glycerol compared to glucose, which is metabolized via the Entner–Doudoroff pathway in pseudomonads (del Castillo et al. 2007; Fuhrer et al. 2005). In addition, the glycerol-associated yield improvement appears to be connected to the evolutionary selection, since no such effect has been observed with the parent strains of \( P. \) \emph{putida} \( S12pal_{xyl}B7 \) (Verhoef et al. 2010). Presumably, the increased PP pathway activity associated with the efficient xylose-utilizing phenotype may allow for a more efficient equilibration of PEP and E4P levels, resulting in more efficient \( p \)-hydroxybenzoate production. It should be noted that the applied proportion of xylose in the feed showed little effect within the range tested, whether the primary substrate was glucose or glycerol. Apparently, the \( p \)-hydroxybenzoate production is not very sensitive to variations in relative xylose concentrations above a certain threshold value.

Unexpectedly, the capacity to transport and/or utilize xylose appeared to be dependent on the primary carbon source. With glycerol, a low concentration of residual xylose was observed that is presumably close to the \( K_m \) of the—yet unidentified—xylose transporter in \( P. \) \emph{putida} \( S12pal_{xyl}B7 \). With glucose as the primary substrate, however, the residual xylose concentrations were higher and furthermore increased more than proportionally with increasing amounts of xylose in the feed. Although this phenomenon is still subject to further study, it may be hypothesized that xylose transport in \( P. \) \emph{putida} \( S12pal_{-xyl}B7 \) is PEP dependent. This would be consistent with the observed increase in residual xylose concentrations with decreasing glucose feed (an already relatively inefficient source of PEP), the relative independency between residual xylose concentration and glycerol feed (a good source of PEP), and the decreased growth rate on xylose when Pal/Tal was introduced (drain on PEP for \( p \)-hydroxybenzoate production). In that case, replacing any PEP-dependent transport systems would be an obvious target for further strain improvement. The GAP/PEP availability may furthermore be improved by constructing an ED-negative, glycolytic \( P. \) \emph{putida} \( S12 \) strain. The contribution of the (ATP-driven) \( E. \) \emph{coli} xylose transporter XylFGH to xylose import was presumably limited as observed previously (Meijnen et al. 2008).

We have demonstrated that \( p \)-hydroxybenzoate production in \( P. \) \emph{putida} can be considerably improved by co-feeding different carbon sources that are metabolized via different, (partly artificial) pathways. Thus, the availability of the key aromatics precursors, PEP and E4P, is improved. In addition to \( p \)-hydroxybenzoate, the production of other aromatic compounds derived from aromatic amino acids may be stimulated via this strategy. Moreover, lignocellulosic hydrolysates, the expected major feedstock for future production of biobased fuels and chemicals (Himmel and Bayer 2009; Kumar et al. 2008; Lange 2007), seems to be ideally suited for aromatics production since glucose and xylose are the predominant constituents. Also the improved production on glycerol presents an additional possibility to deploy a cheap and abundant waste substrate for biocatalytic production of (fine) chemicals.

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