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Bioproduction of *p*-Hydroxystyrene from Glucose by the Solvent-Tolerant Bacterium *Pseudomonas putida* S12 in a Two-Phase Water-Decanol Fermentation▽

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Two solvent-tolerant *Pseudomonas putida* S12 strains, originally designed for phenol and *p*-coumarate production, were engineered for efficient production of *p*-hydroxystyrene from glucose. This was established by introduction of the genes *pal* and *pdc* encoding L-phenylalanine/L-tyrosine ammonia lyase and *p*-coumaric acid decarboxylase, respectively. These enzymes allow the conversion of the central metabolite L-tyrosine into *p*-hydroxystyrene, via *p*-coumarate. Degradation of the *p*-coumarate intermediate was prevented by inactivating the *fcs* gene encoding feruloyl-coenzyme A synthetase. The best-performing strain was selected and cultivated in the fed-batch mode, resulting in the formation of 4.5 mM *p*-hydroxystyrene at a yield of 6.7% (C-mol of *p*-hydroxystyrene per C-mol of glucose) and a maximum volumetric productivity of 0.4 mM h^{-1}. At this concentration, growth and production were completely halted due to the toxicity of *p*-hydroxystyrene. Product toxicity was overcome by the application of a second phase of 1-decanol to extract *p*-hydroxystyrene during fed-batch cultivation. This resulted in a twofold increase of the maximum volumetric productivity (0.75 mM h^{-1}) and a final total *p*-hydroxystyrene concentration of 21 mM, which is a fourfold improvement compared to the single-phase fed-batch cultivation. The final concentration of *p*-hydroxystyrene in the water phase was 1.2 mM, while a concentration of 147 mM (17.6 g $liter^{-1}$) was obtained in the 1-decanol phase. Thus, a *P. putida* S12 strain producing the low-value compound phenol was successfully altered for the production of the toxic value-added compound *p*-hydroxystyrene.

The demand for so called "green" production of chemicals is rapidly increasing due to the declining availability of fossil fuels and the urgency to reduce CO_2 emissions (10, 30). However, this bioproduction may be hindered by the toxicity of the product of interest, such as substituted aromatics, to the production host (1, 2, 12, 29). One way to cope with this product toxicity is to deploy solvent-tolerant microorganisms as biocatalysts (5, 28). Of special interest among these solvent-tolerant hosts are *Pseudomonas putida* strains that have been engineered to produce a variety of compounds such as *p*-hydroxybenzoate (25, 33), *p*-coumarate (19), and (*S*)-styrene oxide (22). In our laboratory, we study and employ the solvent-tolerant *P. putida* S12. This strain is well suited for the production of substituted aromatic chemicals (18, 19, 33, 38) thanks to its extreme solvent tolerance (5, 35) and metabolic versatility toward aromatics (14, 16, 34).

An example of an industrially relevant but extremely toxic aromatic is *p*-hydroxystyrene (4-vinyl phenol) (23). This compound is widely used as a monomer for the production of various polymers that are applied in resins, inks, elastomers, and coatings. Ben-Bassat et al. (2, 3, 23) reported *p*-hydroxystyrene production from glucose in *Escherichia coli*. In this strain, phenylalanine/tyrosine ammonia lyase (PAL/TAL; encoded by *pal*) from *Rhodotorula glutinis* and *p*-coumaric acid decarboxylase (PDC; encoded by *pdc*) from *Lactobacillus plantarum* were introduced for the conversion of L-tyrosine into *p*-hydroxystyrene via *p*-coumarate. The maximum concentration of *p*-hydroxystyrene was limited to 3.3 mM due to the toxicity of the product to the *E. coli* host (3, 23). To alleviate product toxicity, a two-phase fermentation with 2-undecanone as the extractant was performed. This approach resulted in a modest 14.2 mM *p*-hydroxystyrene in the organic phase and 0.5 mM *p*-hydroxystyrene in the water phase (2). Toxicity-related adverse effects on *p*-hydroxystyrene production may also be avoided by dividing the whole process into three stages: production of L-tyrosine from glucose by *E. coli*, conversion of L-tyrosine into *p*-coumarate by immobilized PAL-overexpressing *E. coli* cells, and chemical decarboxylation of *p*-coumarate into *p*-hydroxystyrene (29).

In this report, we address and strongly enhance the bio-based production of *p*-hydroxystyrene from glucose by employing the solvent-tolerant *P. putida* S12 as a host. Previously, two strains, *P. putida* S12 C3 (19) and *P. putida* S12 TPL3 (38), have been constructed for the production of the L-tyrosine-derived aromatics *p*-coumarate and phenol, respectively. These strains were highly optimized for aromatics production, resulting in a heavily increased metabolic flux toward L-tyrosine. Therefore, they are suitable platform strains for the production of other L-tyrosine-derived aromatics (33). The bifunctional

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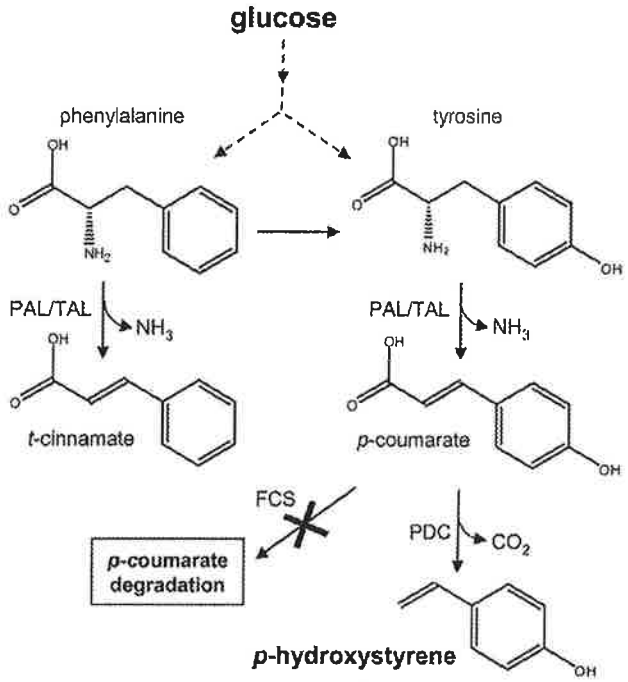

FIG. 1. Schematic overview of the biochemical pathway for *p*-hydroxystyrene production. TAL, tyrosine ammonia lyase; FCS, feruloyl-coenzyme A synthetase. The cross indicates the disruption of *fcs*, disabling *p*-coumarate degradation.

enzyme PAL/TAL (EC 4.3.1.25) from *Rhodosporidium toruloides* and the enzyme PDC (EC 4.1.1.-) from *L. plantarum* were introduced into these strains to allow the conversion of L-tyrosine into *p*-hydroxystyrene (Fig. 1). These minor modifications resulted in an efficient biocatalyst for the production of the value-added compound *p*-hydroxystyrene from glucose.

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) (26) and a phosphate-buffered mineral salts medium, as described previously (9). In the mineral salts medium 20 mM glucose (MMG medium) was used as the sole carbon source. For cultivation of L-phenylalanine auxotrophic strains, 10 mg liter^{-1} L-phenylalanine was added to the medium. The expression of the introduced copy of *aroF-1* in derivatives of *P. putida* S12 strain 427 as well as the expression of *pal pdc* in the pJNTpalpdc vector were induced by addition of 0.1 mM sodium salicylate. Antibiotics were added to the medium as required at the following concentrations: ampicillin, 100 mg liter^{-1} gentamicin, 10 mg liter^{-1} (MMG) or 25 mg liter^{-1} (LB); kanamycin, 50 mg liter^{-1}; and tetracycline, 10 mg liter^{-1} (*E. coli*) or 60 mg liter^{-1} (*P. putida* S12). Shake flask cultivations of *P. putida* S12 were performed in 100-ml Erlenmeyer flasks containing 20 ml of medium in a horizontally shaking incubator at 30°C. Cultures were inoculated with cells from an overnight preculture to a starting optical density at 600 nm (OD_{600}) of approximately 0.2.

Fed-batch experiments were performed in 3-liter fermentors (New Brunswick Scientific) using a BioFlo3000 controller. The initial stirring speed was set to 150 rpm, and air was supplied at 1 liter min^{-1} using Brooks mass-flow controllers (5850 E series and 5850 TR series) and a Brooks 0154 control unit. Dissolved oxygen tension was continuously monitored with an InPro model 6810 probe and maintained at 15% air saturation by automatic adjustment of the stirring speed

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Source or reference
Strains		
P. putida S12	Wild type; ATCC 700801	9
P. putida S12 427[b]	Derived from *P. putida* S12 strain optimized for phenol production	37
P. putida S12 427Δ*fcs*	*fcs* knockout strain of *P. putida* S12 427	This study
P. putida S12 427Δ*fcs* pJNTpalpdc	*fcs* knockout strain of *P. putida* S12 427 containing plasmid pJNTpalpdc	This study
P. putida S12 427Δ*fcs* pJT′Tpalpdc	*fcs* knockout strain of *P. putida* S12 427 containing plasmid pJT′Tpalpdc	This study
P. putida S12 C3	Derived from *P. putida* S12 strain optimized for *p*-coumarate production containing a disrupted *fcs* gene	19
P. putida S12 C3Δ*smo*	*smo* knockout strain of *P. putida* S12 C3	This study
P. putida S12 C3Δ*smo* pJNTpalpdc	*smo* knockout strain of *P. putida* S12 C3 containing plasmid pJNTpalpdc	This study
P. putida S12 C3Δ*smo* pJT′Tpalpdc	*smo* knockout strain of *P. putida* S12 C3 containing plasmid pJT′Tpalpdc	This study
E. coli DH5α	λ$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	26
L. plantarum	Source of *pdc*; DSMZ20174	31
Plasmids		
pTacpal	Ap^r Gm^r; expression vector containing the *pal* gene under the control of the *tac* promoter and *tac* RBS	19
pJT′Tpal	Ap^r Gm^r; expression vector derived from pTacpal containing the *pal* gene under the control of the *tac* promoter and *tac* RBS	Unpublished data
pJNTpal	Ap^r Gm^r; pJNT containing the *pal* gene under the control of the salicylate-inducable *NagR/pNagAa* promoter and *tac* RBS	Unpublished data
pJNTpalpdc	Ap^r Gm^r; pJNT containing the *pal* and *pdc* genes under the control of the salicylate-inducable *NagR/pNagAa* promoter and *tac* RBS	This study
pJT′Tpalpdc	Ap^r Gm^r; expression vector derived from pJT′Tpal containing the *pal* and *pdc* genes under the control of the *tac* promoter and *tac* RBS	This study
pJQ200SK	Suicide vector; P15A *ori sacB* RP4 Gm^r pBluescriptSK MCS	24
pJQfcs::*tet*	pJQ200SK containing the *tetA* interrupted *fcs* gene	19
pJQsmo::*km*	pJQ200SK containing the *km* interrupted *smo* gene	This study

[a] Apr, Gm^r, Km^r, and Tc^r indicate ampicillin, gentamicin, kanamycin, and tetracycline resistance respectively. MCS, multiple cloning site.
[b] Previously known as *P. putida* S12 TPL3c.

TABLE 2. Oligonucleotide primers used in this study

Primer no.	Sequence (5′→3′)[a]	Description
1	GCGGCGGCCGCATGAAAAAGCGTATCGGTATTGTTG	Start of *smo*; forward primer
2	GCGTCTAGATCAATCAGCTCGCCATGCCCTG	Position 569–590 bp in *smo*; reverse primer
3	GCGTCTAGAGAAGTTCTCGCCCACACCAAG	Position 661–681 bp in *smo*, forward primer
4	GCGGGATCCTCAGGCCGCGATAGTCGGTGC	End of *smo*; reverse primer
5	GCGTCTAGAATGAGCCATATTCAACGGGAAACG	Start of Km resistance marker from pTnMod-KmO (6); forward primer
6	GCGTCTAGATTAGAAAAACTCATCGAGCATCAAATG	End of Km resistance marker from pTnMod-KmO (6); reverse primer
7	GCGGCGGCCGCGACATAAGGAAGGTAATTCTAATGAC	Start of *pdc* from *L. plantarum*; forward primer
8	GCGGCTAGCTTACTTATTTAAACGATGGTAGTTTTG	End of *pdc* from *L. plantarum*; reverse primer

[a] Restriction sites are underlined.

and mixing with pure oxygen. The pH was maintained at 7.0 by automatic addition of 4 N NaOH, and the temperature was kept at 30°C. Initial batch fermentation was started with washed cells from an overnight culture in 150 ml of MMG medium with gentamicin and salicylate. The batch phase for both the single- and two-phase fermentations was started with 1.5 liters of medium of the following composition: glucose, 13.5 g; $(NH_4)_2SO_4$, 1.5 g; K_2HPO_4, 5.82 g; $NaH_2PO_4 \cdot H_2O$, 2.44 g; gentamicin, 15 mg; sodium salicylate, 0.15 mmol; and 15 ml of a trace element solution (9). After depletion of the initial ammonium, the feed was started. The feed for the single-phase fermentation contained the following (liter^{-1}): glucose, 158 g; $(NH_4)_2SO_4$, 16.5 g; K_2HPO_4, 3.88 g; $NaH_2PO_4 \cdot H_2O$, 1.63 g; gentamicin, 10 mg; sodium salicylate, 1 mmol; $MgCl_2$, 1.5 g; and 0.1 liter of a trace element solution. The feed for the two-phase fermentation contained the following (liter^{-1}): glucose, 316 g; $(NH_4)_2SO_4$, 33 g; gentamicin, 10 mg; sodium salicylate, 1 mmol; $MgCl_2$, 3 g; and 0.2 liters of a trace element solution. Samples were drawn during the culture to determine the OD_{600} and concentrations of ammonium, glucose, *p*-coumarate, *p*-hydroxystyrene, and *t*-cinnamate.

Analytical methods. Cell densities were determined at 600 nm with an Ultrospec 10-cell density meter (Amersham Biosciences). An OD_{600} of 1 corresponds to 0.49 g liter^{-1} of cell dry weight (CDW). Glucose and organic acids were analyzed by ion chromatography (Dionex ICS3000 system) as described by Meijnen et al. (15). The *p*-coumarate, *p*-hydroxystyrene, and *t*-cinnamate concentrations were analyzed by high-performance liquid chromatography (Agilent 1100 system) using a Zorbax SB-C_{18} column (length, 5 cm; inside diameter, 4.6 mm; particle size, 3.5 μm) and a diode array detector. For analysis of aromatic compounds in aqueous solutions, 25% of acetonitrile in KH_2PO_4 buffer (50 mM, pH 2, 1% acetonitrile) was used as eluant at a flow of 1.5 ml min^{-1} for 4.5 min. For analysis of *p*-hydroxystyrene in 1-decanol, the eluant was 50% acetonitrile in MilliQ at a flow of 1.5 ml min^{-1} for 2.5 min. The samples of *p*-hydroxystyrene in the 1-decanol phase were diluted 100-fold in acetonitrile before analysis. The total *p*-hydroxystyrene concentration in the two-phase fed-batch cultures was calculated as follows (36): $c_{tot} = (c_{aq} \times V_{aq} + c_{dec} \times V_{dec}) \times V_{tot}^{-1}$, where c_{tot}, c_{aq}, and c_{dec} are, respectively, the total *p*-hydroxystyrene concentration and the concentrations in the aqueous and the 1-decanol phases. V_{tot}, V_{aq}, and V_{dec} are, respectively, the total liquid volume and the volumes of the aqueous and the 1-decanol phases.

DNA techniques. Plasmids were introduced into *P. putida* S12 by electroporation using a Gene Pulser electroporation device (Bio-Rad). The targeted gene disruption of the *fcs* gene in *P. putida* S12 strain 427 was performed as described by Nijkamp et al. (19). The gene replacement vector for the *smo* gene, pJQsmo::*km*, was created from pJQ200SK (24) with primers 1 to 4 listed in Table 2, and gene replacement was performed as described previously (33). The kanamycin resistance gene was amplified from plasmid pTnMod-KmO (6) using primers 5 and 6 (Table 2). pJQsmo::*km* was introduced in *P. putida* S12 C3 by triparental mating using *E. coli* HB101 RK2013 (8) as the mobilizing strain and established procedures (7). Cells were plated on *Pseudomonas* isolation agar (Difco) containing tetracycline and kanamycin. Colonies that were kanamycin resistant and gentamicin sensitive were selected. Replacement of the native *smo* gene by a *smo* gene disrupted by the kanamycin resistance marker was confirmed by screening the colonies on LB-agar plates containing 1 mM indole in the presence of air saturated with styrene for styrene monooxygenase (SMO) induction. Native SMO converts indole into indigo, resulting in blue colonies; colonies with inactivated SMO remain white (20).

The *pdc* gene was amplified from genomic *L. plantarum* DNA by PCR using primers 7 and 8 (Table 2), which were designed based on the publicly available *pdc* sequence (GenBank accession no. U63827) (4). Restriction sites NotI and NheI were added for cloning purposes. The *pdc* gene was cloned into pJT′Tpal or pJNTpal, resulting in pJT′Tpalpdc and pJNTpalpdc, respectively.

RESULTS

Degradation of *p*-hydroxystyrene by *P. putida* S12. *P. putida* S12 is able to utilize styrene as a sole carbon source (9). The first step in styrene degradation is the oxidation to styrene oxide by SMO. The encoding *smo* gene is induced by styrene (21, 27). The ability to degrade *p*-hydroxystyrene in the presence or absence of styrene was assessed in *P. putida* S12 and in the *smo*-negative mutant *P. putida* S12 C3Δ*smo*. Wild-type *P. putida* S12 degraded *p*-hydroxystyrene only in the presence of styrene. *P. putida* S12 C3Δ*smo* did not degrade *p*-hydroxystyrene, in either the presence or absence of styrene. These results suggest that *p*-hydroxystyrene is oxidized by SMO and that *p*-hydroxystyrene is not an inducer for *smo*. Therefore, deletion of the *smo* gene is not required for stable *p*-hydroxystyrene production in *P. putida* S12. This was confirmed by the observation that *p*-hydroxystyrene was never degraded in the production experiments with strain 427-derived constructs, which have an intact *smo* gene (data not shown).

Construction of *p*-hydroxystyrene producing *P. putida* S12 strains. Two different strains with an enhanced flux toward L-tyrosine, *P. putida* S12 C3 and *P. putida* S12 427, were modified to produce *p*-hydroxystyrene via L-tyrosine. Since *p*-coumarate is the direct precursor for *p*-hydroxystyrene, the degradation of *p*-coumarate via the *p*-coumarate catabolic pathway should be blocked for optimal *p*-hydroxystyrene production. To this end, the first gene of the *p*-coumarate degradation pathway, *fcs* (encoding feruloyl-coenzyme A synthetase), was inactivated by homologous recombination in *P. putida* S12 427 (Table 1), similar to the inactivation of *fcs* in *P. putida* S12 C3 (19). The *pal* and *pdc* genes were introduced in strains *P. putida* S12 C3Δ*smo* and *P. putida* S12 427Δ*fcs* by transformation of one of the *pal-pdc* expression plasmids pJNTpalpdc or pJT′Tpalpdc, enabling the conversion of L-tyrosine into *p*-hydroxystyrene (Fig. 1). Production of *p*-hydroxystyrene by the resulting four different *P. putida* S12 constructs was assessed during growth on MMG medium in shake flask cultures (Table 3).

Both *P. putida* S12 427-derived strains show a higher product-to-substrate yield ($Y_{p/s}$, where *p* is the amount of product

TABLE 3. Characteristics of *p*-hydroxystyrene production by different *P. putida* S12 strains

P. putida strain	Cultivation method	Max pHS concn (mM)[a]	$Y_{p/s}$ (C-mol %)[b]	$r_{p,max}$ (mM h^{-1})[c]
S12 C3Δ*smo* pJT'Tpalpdc	Shake flask	0.35	2.3	0.01
S12 C3Δ*smo* pJNTpalpdc	Shake flask	0.72	4.8	0.03
S12 427Δ*fcs* pJT'Tpalpdc	Shake flask	0.88	5.9	0.1
S12 427Δ*fcs* pJNTpalpdc	Shake flask	1.1	7.4	0.1
S12 427Δ*fcs* pJNTpalpdc	Single-phase fed batch	4.5	6.7	0.4
S12 427Δ*fcs* pJNTpalpdc	Biphasic fed batch	21[d]	4.1	0.75

[a] Max, maximum; pHS, *p*-hydroxystyrene.
[b] Product to substrate yield in C-mol of *p*-hydroxystyrene per C-mol of glucose.
[c] Maximum volumetric production rate.
[d] For the calculation, see Materials and Methods.

and *s* is the amount of substrate) and maximum volumetric production rate ($r_{p,max}$) than the strains derived from *P. putida* S12 C3. The pJNTpalpdc vector enabled better *p*-hydroxystyrene production than the pJT'Tpalpdc vector in both hosts (Table 3). Therefore, *P. putida* S12 427Δ*fcs* pJNTpalpdc was selected for further study.

Production of *p*-hydroxystyrene in a fed-batch fermentation. In order to increase productivity under controlled conditions, the production of *p*-hydroxystyrene by strain S12 427Δ*fcs* pJNTpalpdc was studied in fed-batch cultures (Fig. 2). *p*-Hydroxystyrene accumulated to a maximum concentration of 4.5 mM with a $Y_{p/s}$ of 6.7 C-mol%, a biomass (x) to substrate yield ($Y_{x/s}$) of 50 C-mol%, and a product to biomass yield ($Y_{p/x}$) of 13 C-mol% (Table 3). Only trace amounts of *p*-coumarate were observed, and the by-product *t*-cinnamate accumulated to a final concentration of 0.36 mM. The ammonium concentration increased slightly after the feed was started, likely due to a decreased growth rate caused by increasing *p*-hydroxystyrene toxicity. The feed rate was adapted to keep the nitrogen concentration below 30 mg $liter^{-1}$. At all time points, glucose or its corresponding acid metabolites gluconate and 2-ketogluconate were present in the broth at a minimum total concentration of

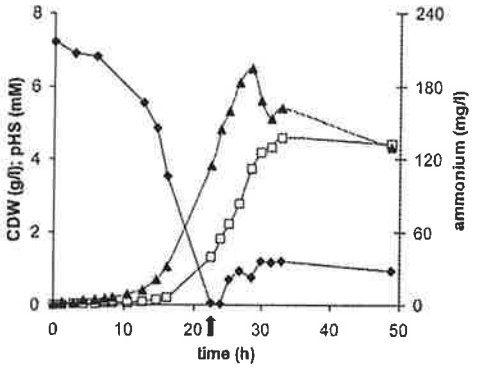

FIG. 2. Production of *p*-hydroxystyrene by *P. putida* S12 427Δ*fcs* pJNTpalpdc in fed-batch cultivation. Concentration *p*-hydroxystyrene (pHS; □), CDW (▲), and ammonium (◆). The arrow indicates the time that the feed was started. The data presented are from a single representative experiment.

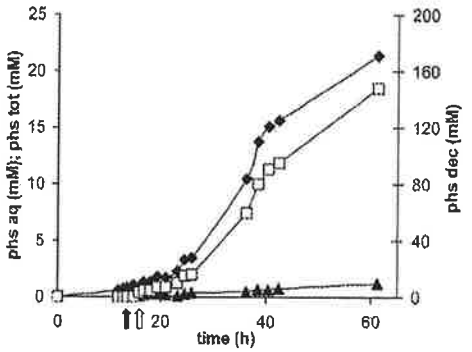

FIG. 3. Production of *p*-hydroxystyrene by *P. putida* S12 427Δ*fcs* pJNTpalpdc during a two-phase water-1-decanol nitrogen-limited fed-batch fermentation. The total concentration of *p*-hydroxystyrene (phs tot [◆]; left *y* axis) calculated as described in the Material and Methods section, concentration of *p*-hydroxystyrene in the 1-decanol phase (phs dec [□]; right *y* axis), and the concentration of *p*-hydroxystyrene in the aqueous phase (phs aq [▲]; left *y* axis) are shown. The filled arrow indicated the time that the feed was started, and the open arrow indicates the time that 1-decanol was added. The data presented are from a single representative experiment.

40 mM. Thus, carbon was always present in large excess to nitrogen.

When a concentration of 4.5 mM *p*-hydroxystyrene was reached, growth and *p*-hydroxystyrene production were completely halted, and lysis occurred (Fig. 2). Twofold dilution of the culture broth with phosphate buffer resulted in a resumption of growth and production until the *p*-hydroxystyrene concentration reached 4.5 mM again (data not shown). These observations suggest that 4.5 mM is a critical concentration that prevents both cellular growth and *p*-hydroxystyrene production.

***p*-Hydroxystyrene production in a nitrogen-limited two-phase water-decanol fed-batch fermentation.** In order to maintain product concentrations below inhibitory levels, biphasic fed-batch cultures were performed with *P. putida* S12 427Δ*fcs* pJNTpalpdc using 1-decanol as the second phase. The conditions were comparable to the single-aqueous phase fermentation except for the addition of 500 ml of 1-decanol after 15 h of cultivation (Fig. 3). Also, the feed was concentrated twofold in order to compensate for the volume that the 1-decanol phase occupies in the bioreactor.

The second phase of 1-decanol maintained the maximum *p*-hydroxystyrene concentration in the water phase (1.2 mM) well below the inhibitory value of 4.5 mM during the fermentation (Fig. 3). The final concentration of *p*-hydroxystyrene in the 1-decanol phase reached 147 mM, and the total *p*-hydroxystyrene concentration was 21 mM at the time when the fermentation was halted (Table 3). Although the *p*-hydroxystyrene concentration was still increasing at the end of the cultivation, the cultivation was stopped due to limitations of the reactor volume (Fig. 3).

The partition coefficient of *p*-hydroxystyrene in this medium/solvent system ($P_{s/w}$) was approximately 120, based on the final concentrations *p*-hydroxystyrene in the aqueous and 1-decanol phases. In the water phase, only trace amounts of *p*-coumarate were detected, and *t*-cinnamate accumulated to a final concentration of 0.55 mM. *p*-Coumarate, *t*-cinnamate, and salicylate

were not extracted by 1-decanol. The growth-limiting compound during fed-batch fermentation (ammonium) was measured to monitor cell growth since the cell growth rate (μ) is related to the ammonium consumption rate. Accumulation of ammonium indicates that the ammonium consumption rate has fallen below the feeding rate, likely due to a decrease in μ caused by *p*-hydroxystyrene toxicity. No ammonium was detected during the feed phase, indicating that *p*-hydroxystyrene did not reach inhibitory concentrations. Glucose, gluconate, and/or 2-ketogluconate was always present throughout the fermentation at a minimum total concentration of 30 mM. The CDW was 2.7 g liter^{-1} when 1-decanol was added. The presence of 1-decanol made further OD_{600} measurements impracticable due to emulsion formation. Therefore, the biomass formation was estimated based on the ammonium consumption, resulting in an estimated final biomass concentration of 30 g liter^{-1}. Based on this estimation, the $Y_{p/x}$ is 13 C-mol%. The $Y_{x/s}$ and $Y_{p/s}$ were 33 C-mol% and 4.1 C-mol%, respectively, assuming that glucose was the sole carbon source. However, in a separate experiment *P. putida* S12 was shown to utilize 1-decanol as a sole carbon source (not shown). Therefore, coutilization of 1-decanol in the biphasic culture cannot be excluded and would serve to reduce the above calculated yield estimates.

DISCUSSION

In the present study, strains *P. putida* S12 427 (37) and *P. putida* S12 C3 (19) have been adapted to produce the L-tyrosine-derived aromatic product *p*-hydroxystyrene. By using these strains as platform hosts, two key issues for the efficient production of toxic aromatics from glucose were addressed. First, these platform strains possess an optimized flux to the central metabolite L-tyrosine, which is a prerequisite for efficient product formation. Second, toxicity of *p*-hydroxystyrene severely limits its production as demonstrated previously (2). This can be averted by using a solvent-tolerant strain such as *P. putida* S12, in combination with in situ product removal using a second phase of extractant.

The biocatalysts derived from strain *P. putida* S12 C3 are L-phenylalanine auxotrophic, resulting in a negligible production of the by-product *t*-cinnamate (19), which is advantageous for downstream processing. However, the auxotrophy strongly affected the growth rate of this strain and therefore also the *p*-hydroxystyrene production rate since growth and production are directly linked. The biocatalysts derived from strain *P. putida* S12 427 show some *t*-cinnamate formation but have a higher growth rate, $Y_{p/s}$ and $r_{p,max}$ than strain C3 derivatives (Table 3). The latter three factors were regarded as more important for efficient production, and thus *P. putida* S12427Δ*fcs* pJNTpalpdc was selected for further study.

Despite the high solvent tolerance of *P. putida* S12, *p*-hydroxystyrene production exerted negative effects on the host cell system. The $Y_{p/s}$ for *p*-hydroxystyrene was approximately 1.5-fold lower than for the less toxic product *p*-coumarate (19; also unpublished data). No accumulation of *p*-coumarate was observed in shake flask cultures, indicating that the conversion of *p*-coumarate into *p*-hydroxystyrene by PDC was not the bottleneck as it was for the *E. coli* production system (23).

In the fed-batch culture of *P. putida* S12 427Δ*fcs* pJNTpalpdc, growth and production were completely inhibited at the critical concentration of 4.5 mM of *p*-hydroxystyrene. Ben-Bassat et al. encountered similar problems with their *E. coli* system but at lower *p*-hydroxystyrene concentrations. To alleviate product toxicity, they added a second phase of 2-undecanone ($P_{s/w}$ of 20) (2, 23), resulting in the production of 14.2 mM *p*-hydroxystyrene in the organic solvent phase. Due to its inherent solvent tolerance, *P. putida* S12 can tolerate both a higher *p*-hydroxystyrene concentration and a second phase of the more efficient extractant 1-decanol ($P_{s/w}$ of 120). This led to the production of 147 mM *p*-hydroxystyrene in the 1-decanol phase, which is a significant 10-fold improvement compared to the *E. coli* system.

Although product toxicity could be substantially alleviated by addition of a second phase of 1-decanol, allowing considerably increased productivity and product titers, a clear negative effect of the extractant phase itself was observed on $Y_{p/s}$. The negative impact of solvents on $Y_{x/s}$ has been extensively studied and can be attributed to both a direct uncoupling effect of the solvent on the proton motive force and the energy demand by RND (resistance-nodulation-cell division)-type solvent extrusion pumps (13, 17). Since biomass and product formation are closely linked, a decreased $Y_{x/s}$ will translate directly into a lower $Y_{p/s}$, which was underlined by the constant $Y_{p/x}$ of 13 C-mol% in both the aqueous and the biphasic water-solvent fed-batch cultivations. The decreased $Y_{x/s}$ caused by the extractant toxicity may be averted by the physical separation of the aqueous and organic phases by using advanced in situ product removal techniques such as solvent-impregnated resins (32) or membrane extraction (11). This, together with a stable genomic integration of the heterologous *pal-pdc* construct, would also lead to a more stable process.

The study presented here clearly demonstrates the usefulness of solvent-tolerant biocatalysts for the production of toxic aromatics in combination with integrated product extraction. By further process optimization of the solvent-to-water ratio, the reactor volume, and the solvent selection, the product titers could be significantly increased. Thus, this work may greatly assist the economically viable production of the value-added chemical *p*-hydroxystyrene from cheap renewable resources, which will be of great environmental as well as economic benefit.

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