Biological production of hydroxylated aromatics

Optimization strategies for *Pseudomonas putida* S12

Suzanne Verhoef

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Biologische productie van gehydroxyleerde aromaten

Strategieën voor optimalisatie van *Pseudomonas putida* S12

Proefschrift

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Anna VERHOEF

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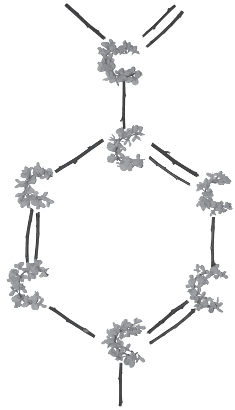

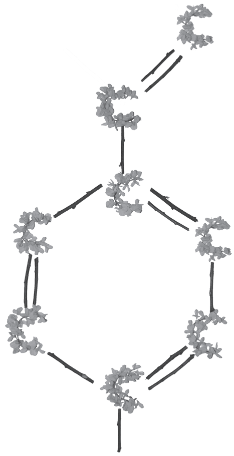

General introduction

In our modern industrialized society petrochemistry is the main supplier of fuels and organic chemicals which are used in a wide variety of products (81). The two organic chemicals that are the focal products of this thesis, *p*-hydroxybenzoate and *p*-hydroxystyrene, are used as monomers for the production of various polymers which are applied in *e.g.* liquid crystals, resins, inks, elastomers and coatings. Furthermore, the pharmaceutical and cosmetic industry applies alkyl ester derivatives of *p*-hydroxybenzoate (parabens) as preservatives. Currently, *p*-hydroxybenzoate and *p*-hydroxystyrene are derived from oil, like the large majority of organic chemicals. The declining availability of fossil resources (8) together with the increasing demand for fossil fuels (7, 25) and the often political instability of oil producing countries (41) have resulted in an increasing demand for suitable alternatives to replace fossil resources. This development is reinforced by world-wide concerns about global warming and asks for sustainable 'green' solutions for the usually environmentally unfriendly chemical processes.

Biological production of chemicals

One way to produce organic chemicals in a sustainable way is the conversion of a renewable substrate into a desired product catalysed by a biological system (2, 20, 81, 100). This type of bio-based conversion, often referred to as biocatalysis, has been applied for many centuries for the production of various fermented foodstuffs like beer, cheese, kimchi and yoghurt. The recent development of genetic engineering techniques has hugely expanded the potential for biological conversions and as result, more and more chemical conversions are being replaced by bio-chemical conversions (77, 83, 100).

In terms of sustainability, the biological production of organic chemicals presents a step forward since carbon dioxide fixation and utilization of the feedstock is much more balanced in time than it is the case for petrochemical synthesis (Fig. 1). The fossil resources utilized in petrochemical synthesis were formed millions of years ago, while their supplies are currently

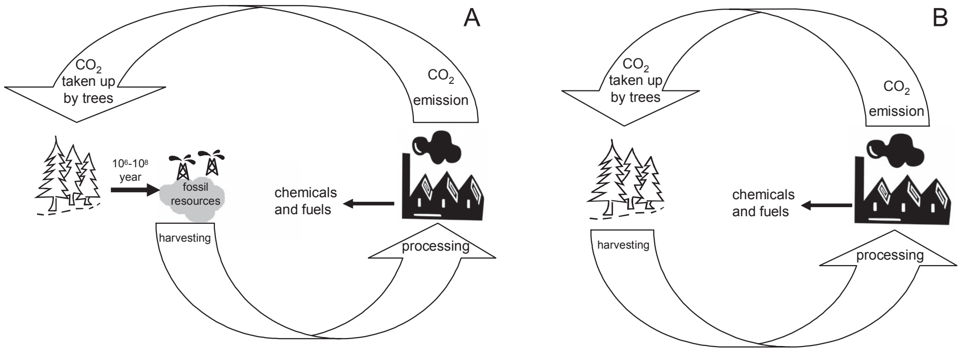

Figure 1. The carbon cycle for fossil resource based products (A) and plant material based products (B) (88).

being exhausted in a very short period. There is a strong concern that the resulting rapid increase of the CO_2 concentration in the atmosphere (7) affects the global climate (39). This can be overcome by using renewable feedstock, *i.e.* biomass, as substrate. The emitted CO_2 is converted into plant material and within a short time reused as feedstock for biological production of fuels and chemicals (Fig. 1B, (88)).

Other advantages of biological production processes are the requirement of usually less toxic chemicals and the possibility to apply relatively mild process conditions. Furthermore, the biocatalyst, substrate and (by-) products are mostly biodegradable (26, 77, 86, 100).

Biocatalytic processes can be divided into two types: biotransformation and bioconversion. In biotransformation, a pre-formed substrate is converted into the desired product by one or a few enzymatic reactions. The required enzymes can be employed as either isolated enzymes or (heterologous) enzymes within a microbial system (83). Unfortunately, the pre-formed substrates may still be produced from fossil resources (24). In bioconversion, the product formation occurs via *de novo* synthesis, *i.e.* the renewable substrate is used for both cell mass as well as the desired product formation. The main challenge of bioconversion reactions is to increase the product-to-substrate yield since a major part of the substrate will be utilized for cell mass formation (14, 58).

Biocatalysis by solvent-tolerant *Pseudomonads*

Bioproduction of various chemicals may be complicated by the toxicity of the product, and in case of biotransformation also of the substrate, to the production host (5, 6, 32, 76). The product / substrate toxicity may be overcome by the use of solvent-tolerant bacteria as biocatalyst (12, 24, 75) since the cellular mechanisms of solvent tolerance can tackle the toxicity of hydrophobic compounds (32, 45, 80). In addition, solvent-tolerant strains commonly show an intrinsic tolerance towards diverse types of aggressive compounds, *e.g.*, not only organic solvents, but also antibiotics and heavy metals (23, 32, 34, 40, 91, 95). This property may contribute to the efficient use of raw (unpurified) bio-based feedstocks.

In 1989 the first solvent-tolerant bacterium was reported (30) and since then, various solvent-tolerant bacteria have been discovered, most of which belong to the genus *Pseudomonas*, especially *P. putida* ((24, 74) and references therein). The mechanisms of solvent tolerance have been extensively studied (for review see (12, 24, 32, 40, 66, 74, 80, 96)) and the main mode of action in response to solvent stress can be summarized as follows:

- Adaptation of the outer cell structure to prevent entry of organic solvents. Gram-negative bacteria can adapt the composition of the lipopolysaccharide layer that surrounds the cells, resulting in increased hydrophilicity and, thus, repulsion of hydrophobic compounds (3, 42, 63). The presence of specific porins in the membrane may also prevent solvent entry by stabilizing the outer cell structure (32, 94).
- Adaptation to the phospholipid bilayer of the cytoplasmic membrane to ameliorate the destabilizing effect of solvents. Accumulation of organic solvents in the cell membrane increases membrane fluidity. This effect is counteracted by making the membrane more rigid, *e.g.* by increasing the *cis-trans* ratio of unsaturated fatty acids (21, 22) and the degree of saturation of the membrane fatty acids (62). Altering the phospholipid headgroup composition (62, 68, 94) also improves membrane stability.

- Active removal of solvents from the cell by means of proton-gradient driven solvent efflux pumps (17, 31, 49, 67, 71).
- Increased energy production to compensate for the energy demand of the solvent efflux pumps as well as for the dissipation of the proton motive force caused by the uncoupling effect of membrane-accumulated solvent molecules (33, 79, 92).

Since solvent-tolerant microbial strains can often tolerate higher concentrations of toxic products (96) this effectively means that a higher final product concentration can be achieved by employing solvent-tolerant biocatalysts in contrast to non-solvent tolerant industrial biocatalysts like *Escherichia coli* (90). Furthermore, the ability of solvent-tolerant bacteria to grow in a second phase of an organic solvent, allows the development of biphasic fermentation setups (24, 78). In such a process, the organic solvent is present as a second phase and acts as a sink for the toxic product/substrate. Consequently, the concentration of the product/substrate remains low in the aqueous phase that contains the bacteria (29, 72, 90, 99). Moreover, since the product accumulates to high concentrations in the organic solvent, *in-situ* product recovery (ISPR) and efficient product purification are highly favoured.

The biocatalytic conversions for which (solvent-tolerant) *P. putida* strains have been employed are mainly focused on the production of aromatics (Table 1), which relates to their extreme tolerance (12, 96) and their metabolic versatility with respect to aromatics (36, 51, 93).

Table 1. Examples of P. putida *based biocatalytic conversions.*

Substrate	Product	Host	Reference
Biotransformations			
Benzene	Catechol	*P. putida* 2313	(70)
Toluene	Toluene *cis*-glycol	*P. putida* UV4	(10)
	p-Hydroxybenzoate	*P. putida* DOT-T1E; *P. putida* EM2878	(47, 65)
	3-Methylcatechol	*P. putida* S12; *P. putida* F1; *P. putida* 2313	(28, 70, 97)
	o-Cresol	*P. putida* T-57	(16)
Limonene	Perillic acid	*P. putida* GS1	(48, 82)
Styrene	(S)-Styrene oxide	*P. putida* SMA	(57)
Testosterone	15-β-Testosterone	*P. putida* S12	(73)
Bioconversions			
Glucose	*t*-Cinnamate	*P. putida* S12	(52)
	Phenol	*P. putida* S12	(99)
	p-Coumarate	*P. putida* S12	(53)
	p-Hydroxybenzoate	*P. putida* S12	This thesis, (89)
	p-Hydroxystyrene	*P. putida* S12	This thesis, (90)
Glycerol	*t*-Cinnamate	*P. putida* S12	(52)
	p-Hydroxybenzoate	*P. putida* S12	This thesis, (89)
Xylose	*p*-Hydroxybenzoate	*P. putida* S12	This thesis

Construction, analysis and optimization of a biocatalyst

Wild type microorganisms are usually inappropriate for the production of organic chemicals at an industrial scale (14, 58) since 1) wild type microorganisms do not contain, or only contain part of, the metabolic pathway required for the production of the compound of interest from a renewable substrate and 2) the metabolic network of microorganisms is not optimized for overproducing that compound. Therefore, the biocatalyst has to be optimized in order to achieve an economically viable process (Fig. 2).

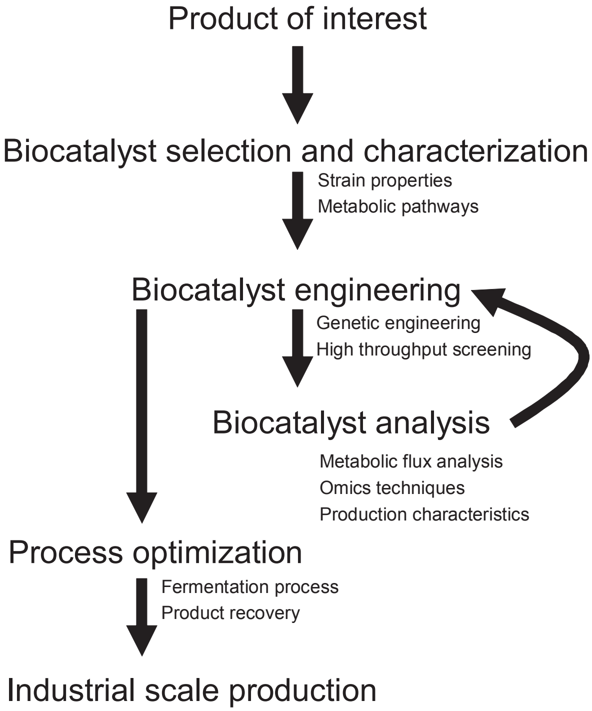

Figure 2. The development of an efficient process for biological production of chemicals. From product of interest towards industrial scale production.

When the microorganism lacks (part of) the metabolic pathway needed for the biosynthesis of the desired compound, heterologous genes can be introduced to complement the missing activities (9, 90). Increasing the product titers usually is a more complex and time consuming process. The major issues to be addressed are product degradation, and an efficient metabolic flux towards the product (89). In general, product degradation can be eliminated by inactivating the first step of the degradation pathway(s) (53, 89). Optimizing the flux towards the product is more challenging: rate limiting steps must be identified and eliminated, and the carbon flow must be directed towards the product, *e.g.*, by eliminating side reactions.

Strains may be optimized via classical approaches such as random mutagenesis followed by selection procedures and / or high throughput screening. Today, strain optimization is further enhanced by modern technologies. Advances in molecular biology, recombinant DNA technology, genetics and genomics technologies have culminated in the availability of modern metabolic engineering technologies that have greatly extended the strain engineering toolbox (1, 14, 96). For the production of chemicals at an industrial scale, the fermentation conditions as well as the product recovery process have to be optimized in addition to the production strains ((58), Fig. 2).

Renewable feedstock

The economic viability of biological production of chemicals is mainly dependent on the feedstock used. Especially in bulk fermentations, overall costs are often dominated by the feedstock costs (61).

Sucrose from sugar beet or sugar cane, and glucose from cereals are two easily fermentable and accessible renewable feedstocks. These are commonly referred to as first generation renewable feedstocks. Due to the rapid growth of, in particular, biofuel production, the demand

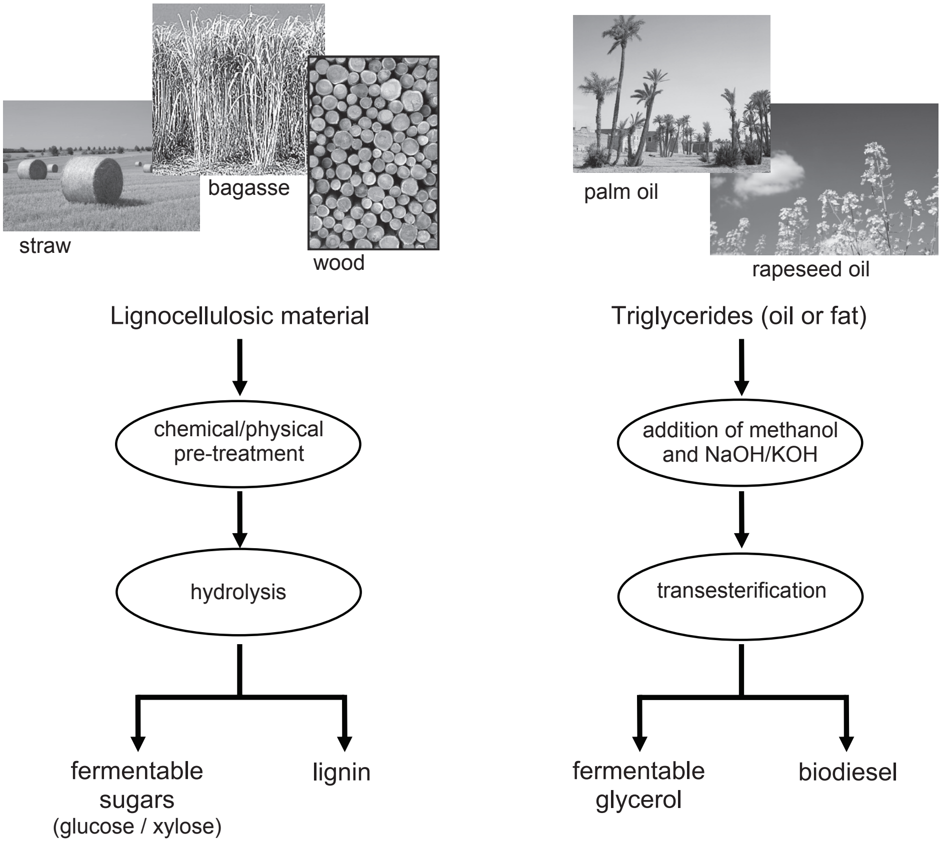

Figure 3. Examples of second generation renewable feedstock and processing.

for sugar has increased sharply and is likely to compete with human food consumption (18, 38). Therefore, a second generation of renewable resources is required that does not have this disadvantage (18).

One of the most abundant renewable resources on earth is lignocellulose. Lignocellulose is composed of cellulose (30-60 %), hemicellulose (20-40 %) and lignin (10-30 %; (43)). In order to convert this material into a fermentable feedstock, the sugars have to be released from the complex of cellulose, hemicellulose and lignin. Acid pre-treatment followed by chemical and/or enzymatic hydrolysis (Fig. 3) is the most commonly used strategy to liberate the sugars (4, 43, 60). The composition of the hydrolysate varies depending on the biomass source, the pre-treatment and the hydrolysis procedures, but the main sugars are glucose (appr. 40 %) and xylose (appr. 15 %; (43)). The appearance of toxic compounds such as furanic aldehydes, organic acids and aromatic compounds during the treatment of lignocellulose material may greatly reduce the fermentability of the hydrolysate (50, 56, 84)

Another promising renewable feedstock is crude glycerol (37). The demand for sustainable fuels resulted in a rapid growth of biodiesel production from vegetable oil. During the production process, glycerol is inevitably formed as the main by-product (Fig. 3; (87)). These two factors resulted in a flooded glycerol marked with decreasing glycerol prices and gave rise to an increased interest in opportunities to convert crude glycerol into higher-value products (11, 37, 55, 102). However, the presence of considerable amounts of impurities in crude glycerol such as methanol, free fatty acids, soap and salts (especially sodium or potassium; (87)) interfere with the fermentation process of various micro-organisms (35, 64, 69) and limit therefore the utility of this renewable fermentation feedstock.

Renewable feedstock as substrate for the production of aromatics by *P. putida* S12

The production of aromatic compounds via the aromatic amino acids tyrosine and phenylalanine has been well established in engineered *P. putida* S12 strains (52, 53, 89, 90, 99). The use of a second generation renewable feedstock, such as crude glycerol or lignocellulose hydrolysate, for the production of these chemicals may have specific advantages.

In fed-batch cultivations of engineered *P. putida* S12 strains on glucose as substrate, the extracellular oxidation of glucose to 2-ketogluconate, via gluconate, hinders an efficient fermentation process. Uptake of 2-ketogluconate is a rate limiting step which reduces growth and therefore aromatics production (44, 52). This problem was circumvented by employing glycerol as substrate (89). The robustness of *P. putida* S12, which tolerates diverse types of aggressive compounds (23, 32, 34, 40, 91, 95), may be exploited to overcome the presence of fermentation inhibitors in crude glycerol (35, 64, 69). Some of the contaminations present in crude glycerol might even be beneficial for aromatics production by *P. putida* S12. Approximately 7 % of the total carbon in crude glycerol consist of long chain fatty acids (87) and can be utilized by *P. putida* strains (27, 85). Consequently, the substrate availability from crude glycerol increases. Furthermore, the high salt concentration in crude glycerol may be beneficial for downstream processing of aromatics through salting-out extraction (54).

Although often regarded as a disadvantage, the mixed composition of hydrolysate may present large benefits to the production of aromatics. The key biosynthetic intermediates for aromatic amino acids are phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P; Fig. 4). The latter compound is an intermediate of the pentose phosphate pathway,

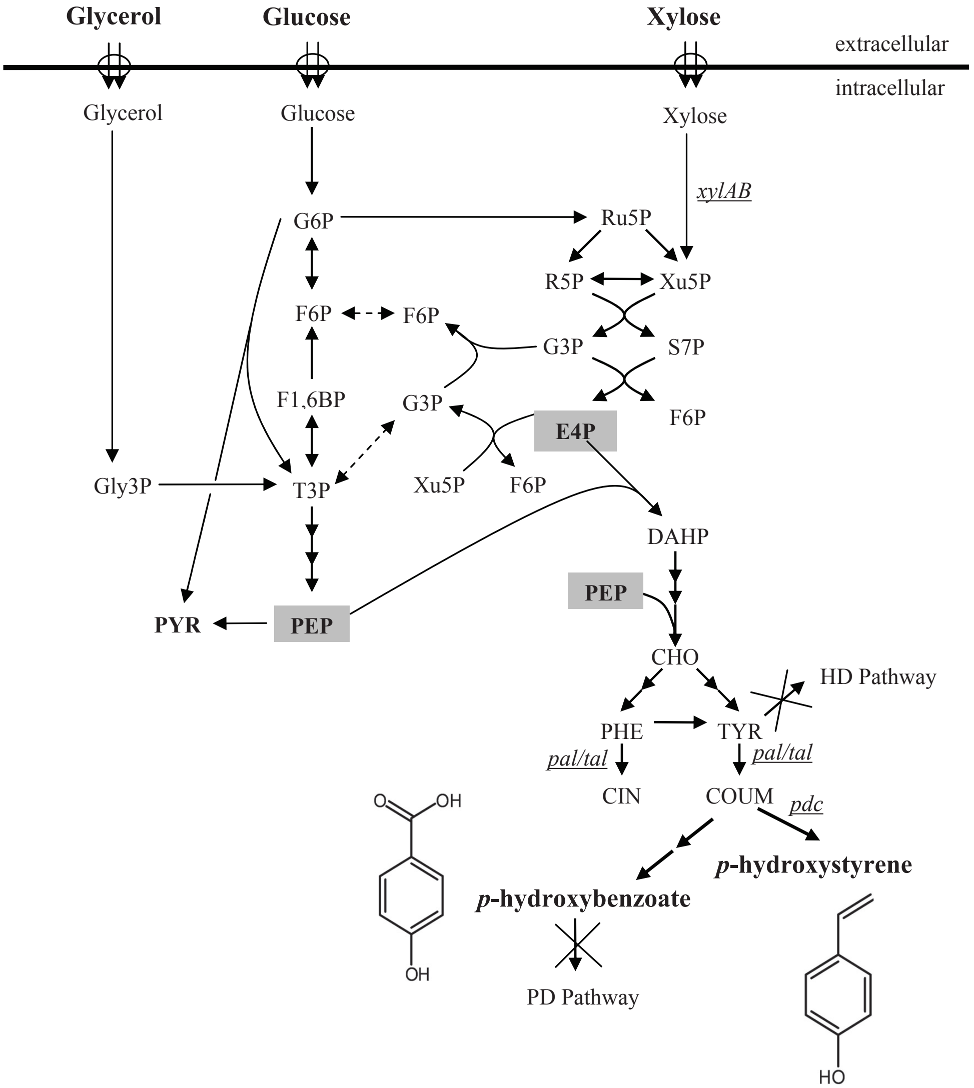

Figure 4. Metabolism of glycerol, xylose and glucose and biosynthesis of p-*hydroxybenzoate and* p-*hydroxystyrene by* P. putida *S12. The scheme shows only relevant routes in which some steps have been simplified. Heterologous genes are indicated in italics and underlined. Xylose isomerase (*xylA*); xylulokinase (*xylB*); phenylalanine/ tyrosine ammonia lyase (*pal/tal*);* p-*coumaric acid decarboxylase (*pdc*); glucose-6-phosphate (G6P); 6-phosphogluconate (6PG); 2-keto-6-phosphogluconate (2-K6PG); 2-keto-3-deoxy-6-phosphogluconate (KDPG); carbondioxide (CO_2); ribulose-5-phosphate (RU5P); xylulose-5-phosphate (X5P); ribose-5-phosphate (R5P); glyceraldehyde-3-phosphate (G3P); sedoheptulose-7-phosphate (S7P); erythrose-4-phosphate (E4P); fructose-6-phosphate (F6P); fructose1,6-bisphosphate (F1,6DP); dihydroxy-acetone-phospahte (DHAP); phosphoenolpyruvate (PEP); pyruvate (PYR); 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP); chorismate (CHO); phenylalanine (PHE); tyrosine (TYR); cinnamate (CIN); p-coumarate (COUM); tricarboxylic acid cycle (TCA cycle); 4-hydroxyphenylpyruvate degradation pathway (HD pathway); protocatechuate degradation pathway (PD Pathway).*

which is nearly inactive in *P. putida* on glucose as substrate (13, 98). The suggested low pool of E4P may therefore pose a bottleneck for aromatics production. The activity of the pentose phosphate pathway can be increased by overexpressing the key genes of the pentose phosphate pathway *tkt* and *tal*, encoding the transketolase and transaldolase (15, 101) and/or by co-feeding pentose sugars directly into the pentose phosphate pathway (19). In view of this, lignocellulose hydrolysate may be an excellent substrate for aromatic production since it contains both hexoses and pentoses (43).

Unfortunately, wild type *P. putida* S12 is not capable of utilizing pentoses. Meijnen *et al* (46) established pentose utilization via the pentose phosphate pathway by introducing the *xylAB* genes (encoding xylose isomerase and xylulokinase) from *Escherichia coli* in *P. putida* S12 (Fig. 4) followed by laboratory evolutionary selection. This resulted in efficient growth and biomass to substrate yield on xylose as well as on arabinose as substrate via the phosphorylative pathway. To prevent oxidation of xylose into xylonate, Gcd activity had to be inactivated (46) which also disabled the unwanted formation of 2-ketogluconate when growing on glucose as substrate.

Scope and outline of this thesis

The investigations described in this thesis were carried out within the B-Basic (Bio-Based sustainable Industrial Chemistry) programme. The main focus of this research programme is the development of new bio-based chemicals from biomass using microorganisms and enzymes as biocatalysts. The goal of this thesis was the biological production of hydroxylated aromatics from renewable feedstock by the solvent-tolerant bacterium *P. putida* S12.

Chapter 1 presents an overview of various aspects of the biological production of chemicals. Special attention was paid to the construction of solvent tolerant biocatalysts as well as to the different types of renewable substrates and their possible effects on aromatics production.

In **Chapter 2**, the construction of a *p*-hydroxybenzoate producing *P. putida* S12 is described. Efficient production via tyrosine was established by overexpressing the heterologous gene *pal/tal* (encoding Pal/Tal; phenylalanine/tyrosine ammonia lyase) and eliminating the degradation of *p*-hydroxybenzoate by targeted disruption of *pobA* (encoding PobA; *p*-hydroxybenzoate hydroxylase) in a *P. putida* S12 strain with an enhanced flux towards tyrosine. The resulting strain was tested for *p*-hydroxybenzoate production from either glucose or glycerol as substrate in shake flask cultures and the product titers were increased by means of a carbon-limited fed-batch cultivation.

In order to investigate the basis of the improved *p*-hydroxybenzoate production and to find leads for further rational strain improvement, -omics techniques were applied on the *p*-hydroxybenzoate producing strain as described in **Chapter 3**. The transcriptomics and proteomics analysis revealed the underlying genetic basic for the increased *p*-hydroxybenzoate production and leads were tested to verify their role in *p*-hydroxybenzoate production **(Chapter 3)**.

The activity of the pentose phosphate pathway is typically low in *P. putida* strains, which suggests that the availability of erytrose-4-phosphate, a key biosynthetic intermediate for aromatic amino acids, may pose a bottleneck for *p*-hydroxybenzoate production. **Chapter 4** describes how the activity of the pentose phosphate pathway in *P. putida* S12 strains can be enhanced. The *p*-hydroxybenzoate producing strain was constructed for xylose utilization and growth characteristics were improved by laboratory evolutionary selection. Carbon limited chemostat cultivations were performed to study the effect of co-feeding xylose to glycerol or glucose on *p*-hydroxybenzoate production.

The economic viability of the biological production of chemicals is mainly dependent on the feedstock used. In **Chapter 5** the utilization of a true industrial-grade renewable substrate, crude glycerol, was demonstrated. The impurities present in crude glycerol strongly interfere with the fermentation process of most micro-organisms. High cell density fed-batch cultivations were performed in **Chapter 5** on both purified and crude glycerol to test whether the impurities have an effect on the growth and production of *P. putida* S12.

In addition to serving as platform strain for the production of *p*-hydroxybenzoate **(Chapter 2)**, *P. putida* S12_427 could easily be transformed to a *p*-hydroxystyrene producing strain as described in **Chapter 6**. For the production of the value-added compound *p*-hydroxystyrene the heterologous enzymes *pal/tal* and *pdc* (encoding Pdc; *p*-coumaric acid decarboxylase) were introduced and *fcs* (encoding Fcs; feruloyl-CoA synthetase) was knocked out. The resulting strain was tested for *p*-hydroxystyrene production in shake flask cultivations. Since *p*-hydroxystyrene is toxic even for the solvent-tolerant *P. putida* S12, a two-phase fed-batch cultivation was performed to alleviate product inhibition and hence, increase the production titers.

Chapter 7 discusses the various optimizations steps for *p*-hydroxybenzoate production described in this thesis and provides suggestions for further strain and process optimization. Furthermore, the relevance of *P. putida* S12 as platform strain for biological aromatics production is evaluated in **Chapter 7**.

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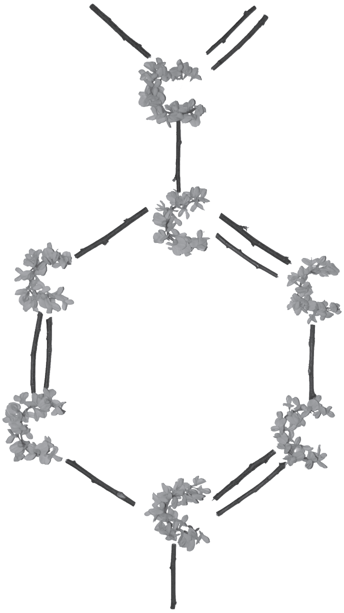
Chapter 2

Bioproduction of *p*-hydroxybenzoate from renewable feedstock by solvent-tolerant *Pseudomonas putida* S12



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Abstract

Pseudomonas putida strain S12palB1 was constructed that produces *p*-hydroxybenzoate from renewable carbon sources via the central metabolite tyrosine. *P. putida* S12palB1 was based on the platform strain *P. putida* S12TPL3, which has an optimized carbon flux towards tyrosine. Phenylalanine/tyrosine ammonia lyase (Pal/Tal) was introduced for the conversion of tyrosine into *p*-coumarate, which is further converted into *p*-hydroxybenzoate by endogenous enzymes. *p*-Hydroxybenzoate hydroxylase (PobA) was inactivated to prevent the degradation of *p*-hydroxybenzoate. These modifications resulted in stable accumulation of *p*-hydroxybenzoate at a yield of 11 % (C-mol C-mol^{-1}) on glucose or glycerol in shake flask cultures. In a glycerol-limited fed-batch cultivation, a final *p*-hydroxybenzoate concentration of 12.9 mM (1.8 g l^{-1}) was obtained, at a yield of 8.5 % (C-mol C-mol^{-1}). A 2-fold increase of the specific *p*-hydroxybenzoate production rate (q_p) was observed when tyrosine was supplied to a steady-state C-limited chemostat culture of *P. putida* S12palB1. This implied that tyrosine availability was the bottleneck for *p*-hydroxybenzoate production under these conditions. When *p*-coumarate was added instead, q_p increased by a factor 4.7, indicating that Pal/Tal activity is the limiting factor when sufficient tyrosine is available. Thus, two major leads for further improvement of the *p*-hydroxybenzoate production by *P. putida* S12palB1 were identified.

Introduction

The fine-chemical *p*-hydroxybenzoate is an industrially relevant compound, which is used as a monomer for the production of liquid crystal polymers (LCP's). Alkyl ester derivatives of *p*-hydroxybenzoate (parabens) are used as preservatives in the pharmaceutical and cosmetic industry. Presently, *p*-hydroxybenzoate is produced almost solely chemically from phenol via the Kolbe-Schmidt reaction (16). This reaction requires extreme temperatures and pressure and a significant volume of by-products are formed. In view of the increasing demand for sustainable processes that utilize renewable feedstock (26, 33), our laboratory focuses on the development of the solvent-tolerant bacterium *Pseudomonas putida* S12 as a platform for the production of aromatic compounds such as phenol (32), *t*-cinnamate (20), *p*-coumarate (21) and *p*-hydroxybenzoate from renewables.

In literature, several biological pathways and production processes for *p*-hydroxybenzoate have been described. The two major routes are the oxidation of toluene into *p*-hydroxybenzoate and the *de novo* bioproduction of *p*-hydroxybenzoate via the intermediate chorismate. The microbial oxidation of the petroleum derivative toluene into *p*-hydroxybenzoate has been described for different *Pseudomonas* species (5, 9, 18). *p*-Hydroxybenzoate can also be produced via the intermediate chorismate, which is converted into *p*-hydroxybenzoate by the enzyme chorismate lyase (UbiC). This pathway has been described in bacteria (*Escherichia coli* (1, 4) and *Mycobacterium tuberculosis* (28)) as well as in plants (sugarcane (17) and tobacco (27)). In *E. coli* *p*-hydroxybenzoate can be produced efficiently from glucose via this pathway (4). However, at higher concentrations *p*-hydroxybenzoate becomes toxic for *E. coli* (4). Solvent-tolerant *P. putida* strains such as S12 are renowned for their extreme tolerance to a variety of chemicals, including aromatics (6, 30) and it would thus be an excellent host for the high level production of *p*-hydroxybenzoate.

Also in *P. putida* S12, *p*-hydroxybenzoate may be produced via the intermediate chorismate. However, this would require the inactivation of all chorismate utilizing enzymes except UbiC (4), resulting in a multi-auxotrophic strain, namely for tryptophan, phenylalanine and tyrosine (8). In order to avoid such multiple auxotrophy and forthcoming difficulties for larger scale cultivations, we followed a different strategy, *i.e.*, production of *p*-hydroxybenzoate via the central metabolite tyrosine. Tyrosine can be converted into *p*-coumarate by expressing phenylalanine/tyrosine ammonia lyase (Pal/Tal) from the yeast *Rhodosporidium toruloides* (20). *p*-Coumarate is further converted into *p*-hydroxybenzoate by the endogenous enzymes *p*-coumaroyl-CoA synthetase (Fcs), *p*-coumaroyl-CoA hydratase/lyase (Ech) and *p*-hydroxybenzaldehyde dehydrogenase (Vdh) (13, 22, 29). In wild-type *P. putida* S12, *p*-hydroxybenzoate is subsequently degraded via the protocatechuate and β-ketoadipate pathway (11, 13, 22).

Previously, a *P. putida* S12 derivative, strain S12TPL3, was constructed for the production of the tyrosine derived product phenol (32). Since the metabolic flux towards tyrosine was greatly improved in this strain, it was expected to be a useful platform host for the production of other tyrosine derived aromatics. Thus, *P. putida* S12TPL3 was used as the parent strain for the construction of a *p*-hydroxybenzoate producing strain that efficiently produced *p*-hydroxybenzoate from sugar or glycerol.

Materials and methods

Bacterial strains, plasmids and culture conditions

The strains and plasmids used in this study are listed in Table 1. *P. putida* S12 was isolated as a styrene-degrading bacterium (10). The media used were Luria broth (LB) (25) and a phosphate buffered mineral salts medium described previously (10). In mineral salts media, 40 mM of glycerol (MMGly) or 20 mM of glucose (MMG) were used as the sole carbon source unless stated otherwise. Antibiotics were added as required to the media at the following concentrations: ampicillin, 100 µg ml^{-1}; gentamicin, 10 µg ml^{-1} (MMG and MMGly) or 25 µg ml^{-1} (LB); kanamycin, 50 µg ml^{-1}; tetracycline, 10 µg ml^{-1} (*E. coli*) or 60 µg ml^{-1} (*P. putida* S12). To induce the expression of the introduced copy of *aroF-1* (32), 0.1 mM of sodium salicylate was added. Shake flask experiments were performed in 250-ml Erlenmeyer flasks containing 50 ml of MMG(ly) in a horizontal shaking incubator at 30°C. Cultures were inoculated to a starting optical density at 600 nm (OD_{600}) of 0.2 with cells from an overnight preculture (MMG) or a 3-day old preculture (MMGly).

Table 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics [a]	Source or reference
Strains		
P. putida S12	Wild type, ATCC 700801	(10)
P. putida S12TPL3	Derived from *P. putida* S12 containing plasmid pNW1, TnAroF-1 transposon and two rounds of NTG mutagenesis followed by *m*-fluoro-D-phenylalanine and *m*-fluoro-tyrosine selection	(32)
P. putida S12TPL3c	*P. putida* S12TPL3 cured from pNW1	Not published
P. putida S12B1	*pobA* knockout strain derived from *P. putida* S12 TPL3c	This study
P. putida S12palB1	*pobA* knockout strain derived from *P. putida* S12 TPL3c containing plasmid pTacpal	This study
P. putida S12pal	*P. putida* S12 containing plasmid pTacpal	(20)
Plasmids		
pTacpal	Ap^r Gm^r , expression vector containing the *pa/tall* gene under control of the *tac* promotor	(21)
pJQ200SK	Suicide vector, P15A *ori sac*B RP4 Gm^r pBluescriptSK MCS	(23)
pJQpobA::tet	pJQ200SK containing the *tetA* interrupted *pobA* gene	This study
pTO1	Tc^r, used for amplification of *tetA*	(14)

[a] Ap^r, Gm^r, Km^r and Tc^r, ampicillin, gentamicin, kanamycin and tetracycline resistance respectively.

Fed-batch experiments were performed in 2-l fermentors (New Brunswick Scientific) using a BioFlo110 controller. Initial batch fermentation was started with washed cells from a 3-day old preculture in 50 ml MMGly. For the batch phase, 1 l of mineral salts medium was used with the following composition (per l): glycerol 4.5 g, $(NH_4)_2SO_4$ 3 g, K_2HPO_4 3.88 g, $NaH_2PO_4 \cdot H_2O$ 1.63 g, gentamicin 10 mg, sodium salicylate 0.1 mmol and 15 ml of trace element solution. The trace element solution contained (per l): Na-EDTA 4 g, $MgCl_2.6H_2O$ 10 g, $ZnSO_4 \cdot 7H_2O$ 0.2 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, $FeSO_4 \cdot 7H_2O$ 0.5 g, $Na_2MoO_4 \cdot 2H_2O$ 0.02 g, $CuSO_4 \cdot 5H_2O$ 0.02 g, $CoCl_2 \cdot 6H_2O$ 0.04 g, $MnCl_2 \cdot 4H_2O$ 0.1 g. After depletion of the initial glycerol, feed 1 and 2 were started. Feed 1 contained (per l): glycerol 90 g, gentamicin 10 mg and sodium salicylate 1 mmol. Feed 2 contained (per l): $(NH_4)_2SO_4$ 132 g, gentamicin 10 mg and 668 ml of trace element solution.

Carbon-limited chemostat cultivations were performed in mineral salts medium containing 20 mM of glycerol, 10 mg l^{-1} gentamicin and 0.1 mM sodium salicylate, in 1-l fermentors (New Brunswick Scientific) with a BioFlo110 controller. Chemostats were inoculated with a 35-ml inoculum of a 3-day old preculture on MMGly. The working volume of the culture was kept at 0.7 l by removing culture broth via a continuously working pump. The dilution rate (D) was set at 0.05 h^{-1} for 36 h, after which it was increased to 0.1 h^{-1}. Cultures were considered to be at steady state when, after at least 50 h of cultivation at D = 0.1 h^{-1} (*i.e.*, 5 volume changes), the glycerol concentration was zero and the *p*-hydroxybenzoate concentration, cell density and stirring speed were constant.

For both fed-batch and chemostat cultures, the initial stirring speed was set to 150 rpm and air was supplied at 1 l min^{-1} using a M+W Instruments D-5111 mass-flow controller. Dissolved oxygen tension was continuously monitored with an InPro model 6900 probe and maintained at 15 % air saturation by automatic adjustment of the stirring speed and mixing with pure oxygen (fed-batch fermentation only). Samples (5 ml for fed-batch, 2 ml for chemostats) were drawn during the culture to determine cell dry weight (CDW), glycerol, ammonium, tyrosine, *p*-coumarate, *p*-hydroxybenzoate and *t*-cinnamate concentrations. CO_2 and O_2 concentrations in the effluent gas were measured using an Innova 1313 Fermentation Monitor. The pH was maintained at 7.0 by automatic addition of 4 N KOH and the temperature was kept at 30°C.

Calculations of the (specific) production rate in pulsed chemostat cultures

To study the effect of tyrosine or *p*-coumarate availability on *p*-hydroxybenzoate production, steady-state chemostat cultures of *P. putida* S12palB1 were pulsed with *p*-coumarate or tyrosine (5 ml of a 350 mM sterile stock solution). The (specific) rates of *p*-coumarate and biomass production were calculated as described below.

The *p*-hydroxybenzoate production rate (r_p) was calculated at each time point (t) according to the formula: $r_{p,t} = C_{p,t} * D * V$ (C-mmol h^{-1}), in which $C_{p,t}$ is the concentration *p*-hydroxybenzoate (C-mmol l^{-1}), D is the dilution rate (0.1 h^{-1}) and V is the volume of the bioreactor (0.7 l).

It was assumed that the culture was at steady-state at each time point with regard to *p*-hydroxybenzoate production, *i.e.*, the amount of *p*-hydroxybenzoate produced ($r_{p,t}$) is equal to the amount of *p*-hydroxybenzoate washed out. The $r_{p,t}$ includes the steady-state production (0.17 C-mmol h^{-1}) as well as the production that results from the pulse. The area under the r_p versus time graph is the total amount of *p*-hydroxybenzoate formed. Subtracting the amount of *p*-hydroxybenzoate formed under steady-state conditions yields the amount of *p*-hydroxybenzoate formed as a result of the pulse. The amount of *p*-coumarate or tyrosine taken up by the cells, and therefore available for the production of *p*-hydroxybenzoate, is the total amount of *p*-coumarate or tyrosine added at t = 0 h minus the amount of *p*-coumarate or tyrosine washed out.

The extra biomass produced as result of the pulse was calculated in a fashion similar to the calculation of *p*-hydroxybenzoate, assuming that 24 g of cell dry weight corresponds to 1 C-mol of biomass (24).

The specific production rate (q_p) was calculated at each time point (t) according to the following formula: $q_{p,t} = (C_{p,t} * D)(C_{x,t} * 60)^{-1}$ (µmol *p*-hydroxybenzoate min^{-1} g cell dry $weight^{-1}$), in which $C_{p,t}$ is the concentration *p*-hydroxybenzoate (µmol l^{-1}), D is the dilution rate (0.1 h^{-1}) and $C_{x,t}$ is the cell dry weight concentration (g l^{-1}).

Analytical methods

Cell densities were determined at 600 nm with a Biowave Cell Density Meter (WPA Ltd). CDW concentrations were calculated from OD_{600} values using the conversion factor CDW (g l^{-1}) = OD_{600} x 0.465. Tyrosine, *p*-hydroxybenzoate, *p*-hydroxybenzaldehyde, *p*-coumarate and *t*-cinnamate concentrations were analyzed by HPLC (Agilent 1100 system) using a Zorbax 3.5 µm SB-C18 column (4.6x50mm) and a diode-array detector. As the eluent, a linear gradient of acetonitrile in KH_2PO_4-buffer (50 mM, pH 2, 1% acetonitrile) was used, increasing from 0-25 % in 4.5 min at a flow of 1.5 ml min^{-1}. Glucose and glycerol concentrations were analyzed by HPLC (Waters) using an Aminex HDP-87N column with 0.01 M Na_2HPO_4 as the eluent and a refractive index detector. NH_4^+ concentrations were determined by cation-exchange chromatography (Dionex) or by an ammonium cuvette test (LCK 303) from Hach Lange GmbH.

DNA techniques

Plasmid pTacpal (21) was introduced into *P. putida* S12 by electroporation using a Gene Pulser electroporation device (Biorad). The suicide vector pJQ200SK (23) was used to construct a gene replacement plasmid for the *pobA* gene as described below. Primers SV1-SV4 (see Table 2 for primer characteristics), based on the *pobA* nucleotide sequence from *P. putida* KT2440 (19), were used to amplify 528 bp of the 5’ end (*pobA*1) and 610 bp of the 3’ end (*pobA*2) of the *pobA* gene. The tetracycline resistance gene (*tet*A) from vector pTO1 (14) was amplified using primers JW5 and JW6 (Table 2). pJQ200SK was digested with *Not*I and *Xho*I and *pobA*1 and *pobA*2 were digested with *Not*I/*Xba*I and *Xho*I/*Xba*I, respectively. The three resulting DNA fragments were ligated to yield pJQpobA. pJQpobA was linearized with *Xba*I and treated with bacterial alkaline phosphatase (BAP). The *Tet*A fragment was digested with *Xba*I and ligated into the linearized pJQpobA vector to yield pJQpobA::tet. This construct was electroporated into *P. putida* S12 TPL3c and cells were plated on LB-agar plates containing tetracycline. Colonies that were tetracycline resistant (Tc^r) and gentamicin sensitive (Gm^s) were selected. Replacement of the native *pobA* gene by the *tet*A disrupted *pobA* copy was confirmed by a colony polymerase chain reaction (PCR) analysis using the primers SV1 and SV4, assessing the inability to grow on *p*-hydroxybenzoate as the sole carbon source and by production of *p*-hydroxybenzoate after introducing pTacpal.

Table 2. Oligonucliotide primers used in this study

Primer	Sequence (5’ → 3’)[a]	Characteristics
JW5	gcgtctagaactcaggtcgaggtggcccgg	Start of *tetA* from pTO1(14), forward primer, *Xba*I
JW6	gcgtctagagaattctcatgtttgacagcttatc	End of *tetA* from pTO1(14), reverse primer, *Xba*I
SV1	gcgcggccgcatgaaaactcaggttgcaattattgg	Start of *pobA*, forward primer, *Not*I
SV2	gcgtctagactgtttcagcacgccctccggg	Pos. 528-507 bp in *pobA*, reverse primer, *Xba*I
SV3	gcgtctagacgccagtcaatcacgagttgatc	Pos. 578-600 bp in *pobA,* forward primer, *Xba*I
SV4	gggctcgagtcaggcaacttcctcgaacggc	End of *pobA*, reverse primer, *Xho*I

[a] Restriction sites are underlined.

Results

Construction of a p-*hydroxybenzoate producing* P. putida *S12 strain*

One of the main issues to be addressed in order to achieve *p*-hydroxybenzoate overproduction from glucose or glycerol was to increase the carbon flux from these substrates towards the central metabolite tyrosine. In our laboratory, the phenol-overproducing strain *P. putida* S12TPL3 had previously been constructed (32) and transcriptomics analyses indicated an increased metabolic flux towards tyrosine (31). Therefore, this strain was expected to be a suitable platform host for the production of other tyrosine-derived aromatics such as *p*-coumarate and *p*-hydroxybenzoate.

First, strain S12TPL3 was cured from plasmid pNW1 (32), yielding strain *P. putida* S12TPL3c devoid of tyrosine phenol lyase activity. This strain no longer produced phenol from glucose (not shown). The enzyme Pal/Tal was introduced in S12TPL3c by transformation of Pal/Tal expression plasmid pTacpal (21). Pal/Tal catalyzes the conversion of tyrosine and phenylalanine into *p*-coumarate and *t*-cinnamate, respectively (20). The strain thus obtained transiently accumulated minute quantities of *p*-coumarate, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate. In addition, *t*-cinnamate accumulated to a final concentration of 712 µM (data not shown). This is in agreement with the previous observations that *t*-cinnamate cannot be degraded by *P. putida* S12 (20), whereas *p*-coumarate is completely metabolized via *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate (13, 22, 29).

To prevent the degradation of *p*-hydroxybenzoate, the *pobA* gene encoding *p*-hydroxybenzoate hydroxylase (13), was replaced with a *tetA*-disrupted copy. Double-crossover recombinants were confirmed by PCR to carry a *tetA*-disrupted copy of *pobA* and to be unable to utilize *p*-hydroxybenzoate. A single clone was pure-cultured and transformed with pTacpal yielding strain *P. putida* S12palB1.

Production of p-*hydroxybenzoate by* P. putida *S12palB1 in shake flask cultures*

The Pal/Tal expressing *pobA* deficient mutant *P. putida* S12palB1 was assessed for *p*-hydroxybenzoate production in shake flasks on mineral salts medium with glycerol (MMGly) or glucose (MMG) (Fig. 1). The lag time for growth on glycerol was longer than for glucose, but the maximum growth rates and biomass yields were comparable for both substrates. The amount of *p*-hydroxybenzoate produced, the maximum specific production rate ($q_{p,max}$) and

Table 3. Characteristics of p-*hydroxybenzoate production by* P. putida *S12palB1*

Medium	Cultivation	Max [pHB] (mM)	Y_{ps} (Cmol%)	$q_{p,max}$ (µmol min^{-1} g CDW^{-1})
MMGly	Shake flask	1.72	10.7	3.6
MMG	Shake flask	1.85	11.0	3.7
MMGly	C-limited fed-batch	12.9	8.5	2.8
MMGly	C-limited chemostat	0.4[a]	4.1[a]	1.0[a]

MMGly Mineral salts medium glycerol, MMG Mineral salts medium glucose, Max [pHB] maximum concentration *p*-hydroxybenzoate, Y_{ps} yield in Cmol *p*-hydroxybenzoate per Cmol glycerol or glucose used x 100%, $q_{p,max}$ maximum specific production rate in µmol *p*-hydroxybenzoate per minute per g cell dry weight (CDW).

[a] at steady-state conditions before addition of the *p*-coumarate or tyrosine pulse respectively.

the overall product yield on substrate (Y_{ps}) were all similar for both substrates (Table 3). *t*-Cinnamate accumulated to a final concentration of 0.1 mM (Fig. 1). During the first hours, transient *p*-coumarate accumulation was observed for both carbon sources. However, the *p*-coumarate accumulation was 3 times higher (up to 350 µM) during growth on glucose (Fig. 1). As a control, a Pal/Tal expressing wild-type *P. putida* S12 (*P. putida* S12pal) was tested. As expected, no accumulation of *p*-hydroxybenzoate was observed. *t*-Cinnamate accumulated to a final concentration of 71 µM.

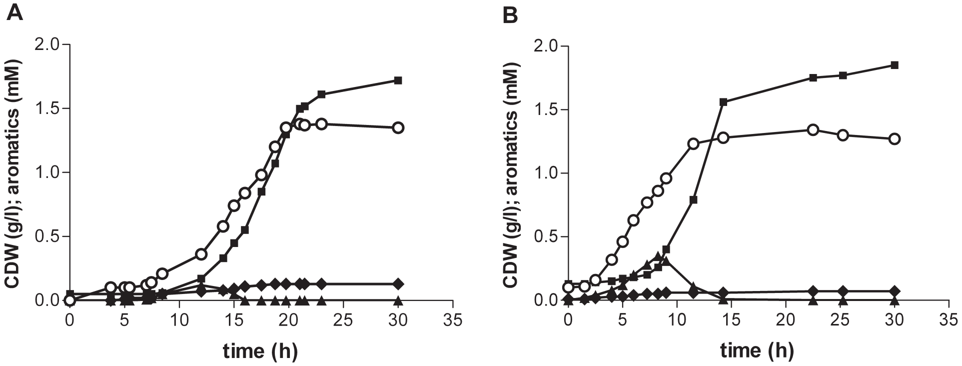

Figure 1. Production of p-*hydroxybenzoate (■),* p-*coumarate (▲),* t-*cinnamate (♦) and biomass (○) by* P. putida *S12palB1 in mineral salts medium with 40 mM glycerol (A) or 20 mM glucose (B) as carbon source in shake flask cultures (in triplicate). CDW: cell dry weight. The data points are the averages of triplicate experiments. The maximum variation between the triplicates was less than 10%.*

Production of p-*hydroxybenzoate by* P. putida *S12palB1 in a carbon-limited fed-batch culture*

The production of *p*-hydroxybenzoate by *P. putida* S12palB1 was studied in fed-batch cultures with glycerol as the limiting substrate to maximize the final concentration of *p*-hydroxybenzoate under controlled conditions. After 29 h of growth, the initially added glycerol was almost depleted and the feeds were started (Fig. 2). Linear growth was maintained by controlling the glycerol feed (feed 1). The ammonium concentration was maintained between 1 and 3 g l^{-1} by controlling feed 2 in order to avoid limitation or accumulation of ammonium and trace elements (data not shown). In the initial batch phase, an extended lag phase was observed in agreement with the shake flask cultures on glycerol. The production of *p*-hydroxybenzoate progressed with growth and stopped almost immediately after growth had ceased (Fig. 2). The fermentation yielded a final concentration of 12.9 mM (1.8 g l^{-1}) of *p*-hydroxybenzoate (Fig. 2). The overall product yield on substrate (Y_{ps}) was 8.5 % (C-mol C-mol^{-1}) and the maximum specific production rate ($q_{p,max}$) was 2.8 µmol *p*-hydroxybenzoate min^{-1} g cell dry $weight^{-1}$ (Table 3). In addition to 12.9 mM of *p*-hydroxybenzoate, 1.1 mM of *t*-cinnamate was co-produced (data not shown). No transient accumulation of *p*-coumarate was observed.

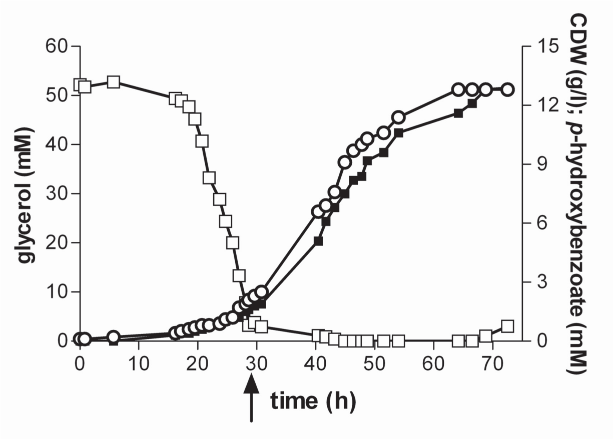

Figure 2. Production of p-*hydroxybenzoate by* P. putida *S12palB1 under carbon limited conditions in fed-batch fermentation. Concentration* p-*hydroxybenzoate (■), cell dry weight (CDW) (○) and glycerol (□).The arrow indicated the time that the feeds were started. The data presented are from a single representative experiment.*

Identification of bottlenecks for p-*hydroxybenzoate production in* P. putida *S12palB1 using pulsed chemostats*

To be able to further improve the product yield of *p*-hydroxybenzoate in *P. putida* S12palB1, the limiting factor in the pathway to *p*-hydroxybenzoate had to be established. Sufficient availability of tyrosine for Pal/Tal is a prerequisite for efficient *p*-hydroxybenzoate production in *P. putida* S12palB1. Although its parent strain S12TPL3 has an increased tyrosine pool, the flux towards tyrosine in *P. putida* S12palB1 may be slower than the conversion of tyrosine into *p*-hydroxybenzoate. Also other tyrosine-utilizing enzymes/pathways may compete with Pal/Tal for tyrosine. In *P. putida* tyrosine can be degraded via the homogentisate pathway (2). Indeed, transcriptome analyses using *P. putida* KT2440-based gene chips (3) on *P. putida* S12palB1 demonstrated that one gene of the homogentisate pathway, 4-hydroxyphenylpyruvate dioxygenase (*hpd*), was 2.8-fold upregulated compared to wild type *P. putida* S12 (data not shown). This observation indicated that tyrosine may be degraded via the homogentisate pathway in *P. putida* S12palB1.

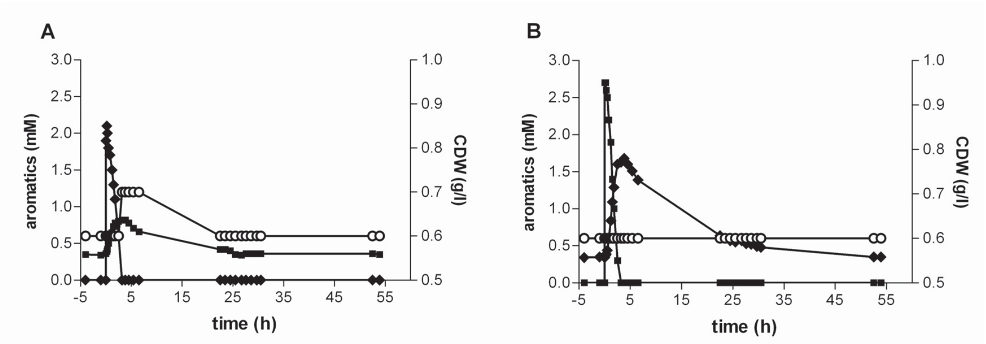

Figure 3. Concentrations of cell dry weight (CDW) (○), p-*hydroxybenzoate (■),* p-*coumarate (▲) and tyrosine (♦) in a carbon limited chemostat culture of* P. putida *S12palB1 in mineral salts medium with 20 mM glycerol. At t=0 h, a pulse of tyrosine (A) or* p-*coumarate (B) was added. The data presented are from a single representative experiment.*

In order to establish whether tyrosine availability was the limiting factor, improved availability was mimicked by the addition of an tyrosine pulse to a C-limited chemostat culture of *P. putida* S12palB1 in steady-state. Upon addition of the pulse, the amount of *p*-hydroxybenzoate increased, as did the amount of cell dry weight (Fig. 3A) and CO_2 (not shown). The *p*-hydroxybenzoate formed above the base level upon addition of tyrosine accounted for 32 % (mol mol $^{-1}$) of the tyrosine added. The increase in cell dry weight and CO_2 corresponded to the remainder of the tyrosine added, plus the amount of acetyl-CoA that is formed in the hydration / two-carbon cleavage step catalyzed by *p*-coumaroyl-CoA hydratase/ lyase in the pathway from *p*-coumarate to *p*-hydroxybenzoate. At steady-state conditions in the chemostat, q_p was 1.0 µmol *p*-hydroxybenzoate min^{-1} g cell dry weight^{-1} (Fig. 4). Upon adding the tyrosine pulse, q_p increased to a maximum of 2.1 µmol *p*-hydroxybenzoate min^{-1} g cell dry weight^{-1} (Fig. 4). Expectedly, pulsing with an equal volume of phosphate buffer had no effect (not shown).

In order to establish whether any rate-limiting steps occurred after tyrosine, *i.e.* in the endogenous conversions of *p*-coumarate into *p*-hydroxybenzoate, a *p*-coumarate pulse was applied in a separate experiment. Addition of *p*-coumarate resulted in an immediate increase of *p*-hydroxybenzoate (Fig. 3B) and CO_2 (not shown), whereas the cell dry weight remained constant (Fig. 3B). It was calculated that the amount of *p*-hydroxybenzoate formed above the base level accounted for 95 % (mol mol $^{-1}$) of the added *p*-coumarate. The extra amount of CO_2 produced was proportional to the amount of acetyl-CoA expected to be formed in the pathway from *p*-coumarate to *p*-hydroxybenzoate. The q_p increased from 1.0 to 4.7 µmol *p*-hydroxybenzoate min^{-1} g cell dry weight^{-1} upon addition of the *p*-coumarate pulse (Fig. 4). The $q_{p,max}$ was apparently not reached as q_p was still increasing when all added *p*-coumarate was depleted (Fig. 4). The uptake rates for *p*-coumarate and tyrosine were similar as derived from Fig 3A and B.

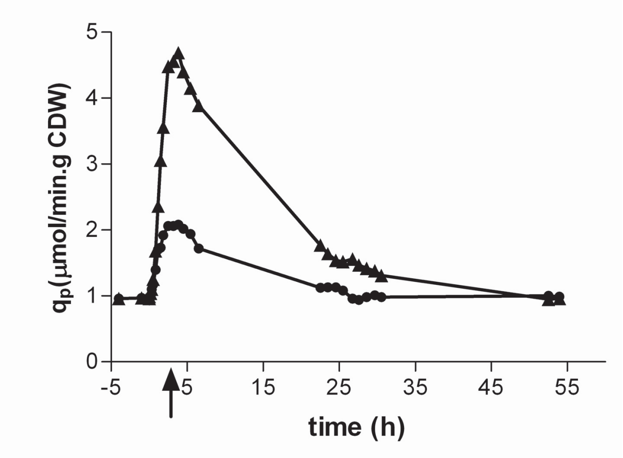

Figure 4. Specific production rate (q_p) of p-*hydroxybenzoate in carbon limited chemostat cultures after a pulse of* p-*coumarate (▲) or tyrosine (●) at t= 0 h. The specific production rate at time point t ($q_{p,t}$; µmol* p-*hydroxybenzoate min^{-1} g cell dry weight^{-1}) was calculated by multiplying the* p-*hydroxybenzoate concentration (µM) at t with the dilution rate (0.1 h^{-1}) divided by the cell dry weight concentration (g l^{-1}) at t. The arrow indicated the time that the concentration of tyrosine, respectively,* p-*coumarate was zero. The data presented are from a single representative experiment.*

Discussion

The bioproduction of phenol (32), *p*-hydroxybenzoate and several other industrially important hydroxylated aromatics from renewable feedstock, such as glucose and glycerol, can proceed via one central metabolite: tyrosine. In such production systems ample availability of tyrosine is imperative for efficient product formation. In the phenol-producing *P. putida* S12TPL3, the metabolic flux towards tyrosine had been increased compared to wild type *P. putida* S12 (32). It was therefore expected that this strain would be a suitable starting strain to construct an efficient *p*-hydroxybenzoate producing host. Indeed *P. putida* S12palB1 accumulated *p*-hydroxybenzoate, with a product yield (Y_{ps}) and a maximum specific production rate ($q_{p,max}$) that were even 1.5-fold higher than for phenol production. Although Tpl (in S12TPL3) and Pal/Tal (in S12palB1) are both inhibited by their reaction products (12, 15), product inhibition is more likely to occur in the phenol producer since phenol is the end product whereas *p*-coumarate is further converted into *p*-hydroxybenzoate.

As expected for products that are derived from central metabolites, the production of *p*-hydroxybenzoate ran parallel to growth. Therefore, a higher final concentration was anticipated in fed-batch cultures, which enable extension of the growth phase. Although the product yield on substrate and the specific production rate were lower than in batch cultures, the final product concentration was improved by a factor 7.

Compared to the *p*-hydroxybenzoate production system via chorismate in *E. coli* as described by Barker and Frost (2001), the product yield (Y_{ps}) in *P. putida* S12palB1 was slightly lower (11 *vs*. 15 % C-mol C-mol^{-1}). However, the production via tyrosine in the solvent-tolerant *P. putida* S12 has several benefits over the production via chorismate in *E. coli*. It is expected that the toxicity of *p*-hydroxybenzoate will limit the maximal attainable product concentrations in *E. coli* sooner than in *P. putida* S12. Barker and Frost (2001) indeed reported that growth and metabolism of a 3-dehydroshikimate-producing *E. coli* strain were substantially inhibited in the presence of 10 g l^{-1} *p*-hydroxybenzoate. Our own findings confirm that *E. coli* is significantly less tolerant for *p*-hydroxybenzoate than *P. putida* S12. When grown in the presence of 7 g l^{-1} *p*-hydroxybenzoate the biomass yield of *E. coli* DH5α decreased by 52 % compared to cultures without *p*-hydroxybenzoate. For *P. putida* S12 (in which *pobA* was knocked out), the decrease in biomass yield was only 20 %. In addition, an optimal production of *p*-hydroxybenzoate via chorismate requires chorismate utilizing enzymes to be blocked. As chorismate serves as the precursor for several essential central metabolites (*e.g.* phenylalanine, tyrosine, tryptophane, ubiquinone and folate) this will result in multiple auxotrophy, which greatly complicates the process. Also post-chorismate products like phenylalanine and prephenate were found with the *E. coli* system as result of nonenzymatic rearrangement of chorismate into prephenate and non enzymatic carboxylation of prephenate into phenylpyruvate which can be converted by a transaminase into phenylalanine (7). This lowered the productivity of the system with 35 % (4). The production of *p*-hydroxybenzoate via tyrosine in *P. putida* S12palB1 does not necessitate the construction of a multi-auxotrophic strain. However, some loss of productivity (5 %) occurs from the formation of *t*-cinnamate from phenylalanine. The production of *t*-cinnamate may complicate product recovery procedures since its physico-chemical features resemble that of p-hydroxybenzoate. This problem can relatively easily be circumvented by constructing a phenylalanine auxotrophic mutant as described by Nijkamp et al. (2007).

In order to further improve the productivity of the *P. putida* system, a clear insight into the bottlenecks in *p*-hydroxybenzoate production is crucial. It appeared that tyrosine availability in *P. putida* S12palB1 was limiting the *p*-hydroxybenzoate production. In chemostat cultures of *P. putida* S12palB1, q_p increased by a factor 2 upon addition of an tyrosine pulse. This demonstrated that, in spite of the improved metabolic flux towards tyrosine in the parent strain, its availability is still a limiting factor for *p*-hydroxybenzoate production. This may (partly) be attributed to tyrosine degradation via the homogentisate pathway, as evidenced from transcriptome analyses showing an upregulation of *hpd*. The first step of tyrosine degradation is deamination to 4-hydroxyphenylpyruvate that is probably catalyzed by several different transaminases. As the biosynthesis of tyrosine also proceeds via 4-hydroxyphenylpyruvate, involving the same enzymes (reverse catabolism), this reaction step can not be blocked. The second step of the degradation pathway is the decarboxylative hydroxylation of 4-hydroxyphenylpyruvate to homogentisate by *hpd* (2). As this conversion is not involved in the production of tyrosine, the availability of tyrosine may be increased by disrupting the *hpd* gene.

It should be noted, however, that tyrosine availability was demonstrated to be a bottleneck under specific conditions, *i.e.*, in a chemostat culture at $D = 0.1\ h^{-1}$. The higher $q_{p,\ max}$ that was attained in batch and fed-batch cultures (3.6 and 2.8 µmol/min/g CDW, respectively) suggested that tyrosine availability may not be the bottleneck under these conditions. A further indication that the system was near saturation with tyrosine also in the chemostat cultures, was the observation that only 32 % of the added tyrosine was converted into *p*-hydroxybenzoate. The remainder was apparently available and, thus, used for growth via the homogentisate pathway. In addition, q_p reached a maximum before all added tyrosine had been taken up by the cell, implying that the pathway from tyrosine to *p*-hydroxybenzoate was indeed saturated. Finally, when the chemostat was pulsed with *p*-coumarate, q_p increased by almost a factor 5 whereas it increased only by a factor 2 with the tyrosine pulse. This indicates that the production of *p*-hydroxybenzoate from *p*-coumarate proceeds 2.5 times faster than from tyrosine. This implies that Pal/Tal-activity will be the next bottleneck for *p*-hydroxybenzoate production when sufficient tyrosine is available. Thus, it is likely that tyrosine availability and Pal/Tal activity are close competitors as limiting factor for *p*-hydroxybenzoate production in *P. putida* S12palB1, and that one prevails over the other depending on the conditions applied.

In conclusion, a *P. putida* S12 strain was constructed that efficiently produces *p*-hydroxybenzoate from renewable carbon sources such as glucose or glycerol via the central metabolite tyrosine. Judging from the dramatic increase in q_p upon external addition of *p*-coumarate, a considerable improvement of the production characteristics is still possible. To this end, two important bottlenecks established in this study should be tackled: Pal/Tal-activity and tyrosine availability. Pal/Tal activity may be improved by increasing expression levels or via directed evolution, both of which approaches are currently pursued. In addition, leads from proteomics and transcriptomics experiments are investigated to further optimize tyrosine availability in strain S12palB1 in a targeted fashion, by improving the metabolic fluxes towards and preventing the degradation of tyrosine and its intermediates.

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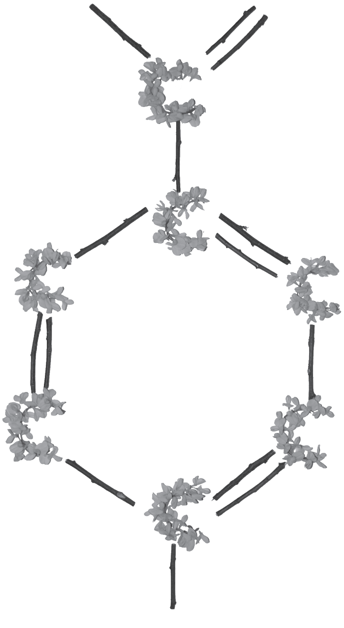
Chapter 3

Comparative transcriptomics and proteomics of *p*-hydroxybenzoate producing *Pseudomonas putida* S12: novel responses and implications for strain improvement



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Abstract

A transcriptomics and proteomics approach was employed to study the expression changes associated with *p*-hydroxybenzoate production by the engineered *Pseudomonas putida* strain S12palB1. To establish *p*-hydroxybenzoate production, phenylalanine/tyrosine ammonia lyase (*pal/tal*) was introduced to connect the tyrosine biosynthetic and *p*-coumarate degradation pathways. In agreement with the efficient *p*-hydroxybenzoate production, the tyrosine biosynthetic and *p*-coumarate catabolic pathways were upregulated. Also many transporters were differentially expressed, one of which -a previously uncharacterized multidrug efflux transporter with locus tags PP1271 - PP1273- was found to be associated with *p*-hydroxybenzoate export. In addition to tyrosine biosynthesis, also tyrosine degradative pathways were upregulated. Eliminating the most prominent of these resulted in a 22-% *p*-hydroxybenzoate yield improvement. Remarkably, the upregulation of genes contributing to *p*-hydroxybenzoate formation was much higher in glucose than in glycerol-cultured cells.

Introduction

The aromatic compound *p*-hydroxybenzoate is a building block of liquid crystal polymers (LCPs), high performance plastics that are employed in electronic devices such as mobile phones. At present, such compounds are derived from fossil resources, but the increasing demand for 'green' polymers and resins requires other approaches to the synthesis of the building blocks that constitute these materials. Biological production from renewable feedstock is a very attractive option, although the hydrophobic nature of aromatic compounds makes specific demands on the production host. Therefore, we have developed a solvent-tolerant bacterium, *Pseudomonas putida* S12, as a host for the biological production of aromatic compounds from renewable resources (9, 10, 20, 21, 26). This organism can tolerate a second phase of aromatic and aliphatic hydrophobic compounds such as toluene and 1-octanol, which property makes this organism an excellent platform host for the production of toxic hydrophobic compounds.

For the production of the tyrosine-derived aromatics phenol, *p*-hydroxystyrene and *p*-hydroxybenzoate, we have exploited *P. putida* S12_427 as a platform strain (Fig. 1; 20, 21, 25). Strain S12_427 was obtained by subjecting wild type *P. putida* S12 to a series of random and targeted optimization steps, key elements in which were the overexpression of the *aroF-1* encoded class-I DAHP synthase, random mutagenesis and antimetabolite screening (Fig. 1; 26). Transcriptomics and metabolic flux analyses on *P. putida* S12TPL3, a phenol producing strain based on *P. putida* S12_427, indicated that the metabolic flux towards tyrosine was significantly improved (24, 25).

For the production of *p*-hydroxybenzoate, tyrosine biosynthesis in *P. putida* S12_427 was connected to the endogenous *p*-coumarate catabolic pathway by introducing the *pal/tal* gene from *Rhodosporidium toruloides* (Fig. 1; 20). This gene encodes phenylalanine/tyrosine ammonia lyase (Pal/Tal) that converts tyrosine into *p*-coumarate. The resulting

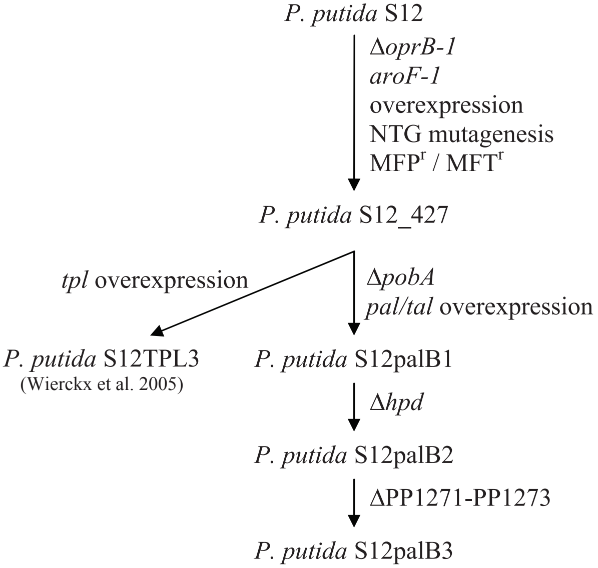

Figure 1. Genealogy of aromatics producing mutants of P. putida *S12. The right-hand column shows how each successive mutant was obtained.* oprB-1*, porin B;* aroF-1*, class-I DAHP synthase;* pobA*, p-hydroxybenzoate hydroxylase;* hpd*, 4-hydroxyphenylpyruvate dioxygenase; PP1271-PP1273, multidrug efflux MFS transporter;* pal/tal*, phenylalanine/tyrosine ammonia lyase;* tpl*, tyrosine-phenol lyase; NTG, N-methyl-N'-nitro-N-nitrosoguanidine; MFP^r: resistant to 100 mg/l m-fluoro-DL-phenylalanine; MFT^r, resistant to 100 mg/l m-fluoro-L-tyrosine.*

synthetic pathway could be employed to accumulate the *p*-coumarate catabolic intermediate *p*-hydroxybenzoate, after eliminating the *pobA* gene encoding *p*-hydroxybenzoate hydroxylase. Fig. 3 presents an overview of *p*-hydroxybenzoate biosynthesis in the resulting *p*-hydroxybenzoate producing strain, *P. putida* S12palB1 (20).

In the present study we employed a transcriptomics and proteomics approach to study the expression changes associated with *p*-hydroxybenzoate production by *P. putida* S12palB1. Thus, insight could be provided into the effects brought about by *p*-hydroxybenzoate accumulation, in conjunction with the impact from the –mostly non-targeted- strain improvement strategy used to obtain the parent strain, *P. putida* S12_427. Based on these insights, new strategies for further –targeted- strain improvement may be designed. Special attention was focused on the pathways directly involved in *p*-hydroxybenzoate production, *i.e.*, aromatic amino acid metabolism and *p*-coumarate degradation. Although previous research suggested no major differences between *p*-hydroxybenzoate production on glucose or glycerol (20), substrate specific effects at the transcript and proteome level cannot be excluded. Especially in view of the artificial connection that was made between an anabolic and a catabolic pathway to achieve *p*-hydroxybenzoate production, carbon source dependent regulatory effects were anticipated. The optimized *p*-hydroxybenzoate production strain *P. putida* S12palB1 was therefore compared to non-optimized control strains on glucose as well as on glycerol.

Material 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) (15) and a phosphate buffered minimal medium adapted from a previously described medium (20) containing 3.88 g l^{-1} K_2HPO_4 and 1.63 g l^{-1} NaH_2PO_4. In minimal media, 40 mM of glycerol (MMGly) or 20 mM of glucose (MMG) were used as the sole carbon source unless stated otherwise. Antibiotics were added as required to the media at the following concentrations: gentamicin, 10 µg ml^{-1} (MMG and MMGly) or 25 µg ml^{-1} (LB); tetracycline, 10 µg ml^{-1} (*E. coli*) or 60 µg ml^{-1} (*P. putida* S12). The expression of the introduced copy of *aroF-1* as well as the expression of the multidrug efflux MFS transporter in the pBNTmfs vector was induced by addition of 0.1 mM of sodium salicylate. Shake flask experiments were performed in 250-ml Erlenmeyer flasks containing 50 ml of MMG or MMGly in a horizontal shaking incubator at 30°C. Cultures were inoculated to a starting optical density at 600 nm (OD_{600}) of 0.2 with cells from a preculture on MMG or MMGly.

Carbon-limited chemostat cultivations were performed as described previously (20) using minimal medium containing either 10 mM glucose or 20 mM glycerol, 10 mg l^{-1} gentamicin and 0.1 mM sodium salicylate, in 1-l fermentors with a BioFlo110 controller (New Brunswick Scientific, Nijmegen, The Netherlands). Chemostats were inoculated with a 35-ml inoculum of a late-log phase preculture on MMGly or MMG. The dilution rate (D) was set at 0.05 h^{-1} for 16 h, after which it was increased to 0.1 h^{-1}. The cultures were considered to be at steady state when no significant changes were measured in cell density, stirring speed and, when applicable, *p*-hydroxybenzoate concentration after at least 5 volume changes at $D = 0.1\ h^{-1}$.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics [a]	Source or reference
Strains		
P. putida S12	Wild type, ATCC 700801	(3)
P. putida S12_427[b]	*P. putida* S12 with an enhanced tyrosine production	(25)
P. putida S12_427 pJTTpal	*P. putida* S12 427 containing plasmid pJTTpal	This study
P. putida S12palB1	*pobA* knockout strain derived from *P. putida* S12 427 containing plasmid pJTTpal	(20)
P. putida S12B2	*pobA* and *hpd* knockout strain derived from *P. putida* S12 427	This study
P. putida S12palB2	*P. putida* S12B2 containing plasmid pJT'Tpal	This study
P. putida S12pal_mfsB2	*P. putida* S12B2 containing plasmids pJT'Tpal and pBNTmfs	This study
P. putida S12B3	Multidrug efflux MFS transporter (PP1271-PP1273) knockout strain derived from *P. putida* S12B2	This study
P. putida S12palB3	*P. putida* S12B3 containing plasmid pJT'Tpal	This study
P. putida S12pal_mfsB3	*P. putida* S12palB3 containing plasmids pJT'Tpal and pBNTmfs	This study
P. putida S12 pJTTpal	*P. putida* S12 containing plasmid pJTTpal	(9)
P. putida S12 pJNNmcs(t)	*P. putida* S12 containing plasmid pJNNmcs(t)	(26)
Escherichia coli DH5α	*supE*44 Δ*lac*U169 (φ80 *lacZ*ΔM15) *hsdR*17 *rec*A1 *endA*1 *gyrA*96 *thi*-1 *relA*1	(15)
Plasmids		
pJTTpal[c]	Ap^r Gm^r, expression vector containing the *pal* gene under control of the *tac* promoter (pALter-EX1, U47102) and the *tac* RBS	(10)
pJT'Tmcs	Ap^r Gm^r, vector for P*tac* and *tac* RBS controlled expression	This study
pJT'Tpal	Ap^r Gm^r, Pal/Tal expression vector under control of P*tac* and *tac* RBS	This study
pJNNmcs(t)	Ap^r Gm^r, vector for P*nagAa* and *nagAa* RBS controlled expression	(26)
pBNTmcs(Gm^r)	Gm^r, vector for P*nagAa* and *tac* RBS controlled expression	This study
pBNTmfs(Gm^r)	Gm^r, multidrug efflux MFS transporter (PP1271-PP1273) expression vector under control of P*nagAa* and *tac* RBS	This study
pJQ200SK	Gm^r, Suicide vector, P15A *ori sac*B RP4 pBluescriptSK MCS	(13)
pJQpobA::tetAloxP	Gm^r Tc^r, pJQ200SK containing the *loxP*-*tetA*-*loxP* interrupted *pobA* gene	This study
pJQhpd::tetAloxP	Gm^r Tc^r, pJQ200SK containing the *loxP*-*tetA*-*loxP* interrupted *hpd* gene	This study
pJQmfs::tetAloxP	Gm^r Tc^r, pJQ200SK containing the *loxP*-*tetA*-*loxP* interrupted multidrug efflux MFS transporter (PP1271-PP1273)	This study

[a] Ap^r, Gm^r and Tc^r, ampicillin, gentamicin and tetracycline resistance respectively.

[b] Previously known as *P. putida* S12 TPL3c.

[c] Previously known as pTacpal.

Sampling for microarray and 2D-DIGE *analysis*

Samples were drawn for microarray and 2D-DIGE analysis from chemostat cultures at steady state conditions. OD_{600} was measured and aromatics and carbon source concentrations were analyzed in the sample supernatants. For each condition (Table 3), three separate chemostat cultivations were performed. For microarray analysis, samples were quenched in ice-cold methanol and centrifuged. The pellet was covered by 1 ml RNAlater (Ambion, Foster City, CA, USA) and incubated for at least 1 h at 4 °C, after which the RNAlater was removed. The pellet was flash frozen in liquid nitrogen and stored at -80 °C. Samples for proteomics analysis were centrifuged at 4000 rpm for 20 minutes at 2 °C. The pellet was flash frozen in liquid nitrogen and stored at -80 °C.

mRNA isolation and cDNA preparation for microarray analysis

Messenger RNA (mRNA) was isolated from the frozen pellets as described by Wierckx et al. (25). Random priming, cDNA synthesis, purification, fragmentation, and labelling were performed according to the microarray manufacturer's instructions (Affymetrix, Santa Clara, CA, USA).

Microarray analysis

High-density custom microarrays based on the genome of *P. putida* KT2440 with additional probe sets were used for transcriptomics analysis (1, 25). The end-labelled cDNA fragments were hybridized to the microarray according to standard manufacturer's protocols. Hybridized microarrays were scanned by ServiceXS (Leiden, The Netherlands) on a high resolution Gene Chip Scanner 3000 7G system with autoloader (Affymetrix, Santa Clara, CA, USA) using standard default analysis settings (filter: 570 nm; pixel size: 2,5 µm). The resulting microarray data (.cel files) were imported into Genespring GX software package version 7.3.1 (Agilent Technologies, Santa Clara, CA, USA) using the GC RMA algorithm. After normalization (signals below 0.01 were taken as 0.01; per chip: normalise to 50th percentile; per gene: normalise to specific sample), probesets representing intergenic regions and control genes were removed, as well as nonchanging genes (between 0.667- and 1.334-fold change). On the resulting set of differentially expressed genes a 1-way ANOVA test (parametric test, *p*-value of 0.05) was applied. Genes that were differentially expressed by at least a factor 2.5 were selected for further analysis.

Protein extraction

Protein extracts were prepared as described by Wijte et al. (27). In brief, cells were boiled for 10 min in a Tris-SDS solution (0.2 % SDS, 0.028 M Tris-HCl, 0.022 Tris-base, and 0.2 M DTT) and subsequently a DNase/RNase treatment was given. Proteins were then extracted by the methanol/chloroform method. The protein pellet was solubilized in a solution with 8 M urea, 4 % CHAPS and 30 mM Tris and sonicated on ice (4 times 5 s). Protein concentrations were determined with the RC/DC assay (BioRad, Veenendaal, The Netherlands).

2D gel electrophoresis

Proteomics analysis was performed using the 2D-DIGE (two dimensional difference-in-gel-electrophoresis) method, according to Volkers et al. (23) and Wijte et al. (27). Protein samples were labeled according to the manufacturers' protocol (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with CyDyes Cy3 and Cy5. The standard sample, consisting of a mixture of equal amounts of all samples in the experiment, was labeled with Cy2. First dimension electrophoresis was performed according to the manufacturers' protocol (GE Healthcare). In short, the labeled proteins were applied to a 24 cm Immobiline Dry-Strip pH 4-7 and rehydrated overnight or applied with cup-loading to a pre-hydrated 18 cm Dry-Strip pH 6-11. Iso-electric focusing was carried out using an IPGphor for a total of 60750 Vh for the pH 4-7 strip and 30750Vh for the pH 6-11 strip. Prior to the second dimension electrophoresis, the strips were refocused for a total of 2500 Vh. After that, the strips were incubated in equilibration buffer containing 1 % (w/v) DTT for 15 min, followed by a 15-min incubation in equilibration buffer containing 2.5 % (w/v) iodoacetamide. After equilibration, the strips were placed on top of 12.5 % polyacrylamide lab-cast gels (pH 4-7) or 15 % polyacrylamide lab-cast gels (pH 6-11) and sealed with a 1 % agarose solution containing a trace of bromophenol blue. Gels were run for 1 h at 1 W per gel followed by 13 W per gel until the bromophenol blue had migrated to the bottom of the gel.

Image acquisition and data analysis

The gels were scanned using a Typhoon 9400 Imager at 100 µm resolution, according to the manufacturers' protocol (GE Healthcare). Data analysis was performed using DeCyder 2D Software version 6.5 with the DeCyder Extended Data Analysis module version 1.0 (both GE Healthcare). After one-way analysis of variance (Student's *t*-test with a *p*-value of 0.05), significantly changed proteins were selected that had an average fold-change difference of at least 1.5 between the conditions tested.

In-gel digestion and protein identification

Preparative polyacrylamide gels were prepared following the protocol for analytical gels described above, with the following changes: only the Cy2-labeled standard was applied and after scanning the gels were stained with ProteomIQ Blue according to the manufacturer's instructions (Proteome Systems, Woburn, MA, USA). After excision, the proteins spots were digested in-gel with trypsin, using the protocol described by Havlis et al. (4). Protein identification was performed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry and microliquid chromatography electrospray tandem mass spectrometry (µlC-ESI MS/MS), as described previously (23).

Analytical methods

Cell densities were determined at 600 nm with an Ultrospec 10 cell density meter (GE Healthcare). An OD_{600} of 1 corresponds to 0.49 g liter^{-1} of cell dry weight (CDW). The aromatic compounds were analyzed by HPLC (Agilent 1100 system) using a Zorbax 3.5 µm SB-C18 column (4.6 x 50 mm) and a diode-array detector. As the eluent, a linear gradient of acetonitrile in KH_2PO_4-buffer (50 mM, pH 2) with 1 % acetonitrile was used, increasing from 0-25 % in 4.9 min at a flow of 1.5 ml min^{-1}. Glucose, gluconic acid and 2-ketogluconic acid were analyzed as described by Meijnen et al (7). Glycerol was analyzed by ion chromatography (Dionex ICS3000 system), using an IonPac ICE AS1 column with 100 mM methyl sulphonic acid as the eluent at a flow of 1.2 ml min^{-1}.

Targeted gene disruption

The previously employed gene replacement vectors pJQpobA::tetA (20) and pJQhpd::

tetA (25) were modified by replacing the *tetA* marker by the same gene flanked by *loxP* recombination sites (16, 17) yielding pJQpobA::tetAloxP and pJQhpd::tetAloxP (Table 1). For construction of the gene replacement vector pJQmfs::tetAloxP, two parts of the multidrug efflux MFS transporter genes (locus tag PP1271-PP1273) were amplified by PCR using primers NW23-NW26 (Table 2). The resulting PCR products mfs1 (5' end) and mfs2 (3' end) were digested with NotI/XbaI and XhoI/XbaI, respectively. Suicide plasmid pJQ200SK (13) was digested with NotI and XhoI. The three resulting DNA fragments were ligated to yield pJQmfs. pJQmfs was linearized with XbaI and treated with bacterial alkaline phosphatase (BAP). The XbaI fragment from pSKtetlox (unpublished data) containing the *loxP* flanked *tetA* marker (16, 17) was ligated into XbaI-digested pJQmfs yielding pJQmfs::tetAloxP.
Targeted gene disruptions were performed as described by Verhoef et al. (20). The *loxP*-flanked *tetA* marker was removed by site-specific recombination after introducing pJNTcre (unpublished data) and induction of the Cre recombinase with 0.1 mM Na-salicylate. Colonies that were tetracycline sensitive (Tc^s) were selected and plasmid pJNTcre was removed by overnight culturing in non-selective Luria broth. Replacement of the native gene by the disrupted copy was confirmed by a colony PCR.

Construction of expression plasmids

Plasmid pBNTmcs(Gm^r) was constructed as follows. The chloramphenicol (Cm) marker from pBBR1mcs (6) was amplified by PCR using the primers Cm_F-AvaI and Cm_R_MluI (Table 2). The resulting fragment was ligated into the pJNTmcs (7) vector using the restriction sites MluI and Kpn2I (compatible with AvaI). From this vector, the salicylate-inducible *NagR/*

Table 2. Oligonucleotide primers used in this study.

Primer	Sequence (5' → 3')[a]	Characteristics
NW23	CGGATCCTGGCGACACCTCGCACAA	start of PP1271 (multidrug efflux MFS transporter), forward primer, BamHI
NW24	GCTCTAGATCAAGGGTCCATCCTGCC	end of PP1271 (multidrug efflux MFS transporter), reverse primer, XbaI
NW25	GCTCTAGAAAACCGGGATTACGCTTGAG	start of PP1273 (multidrug efflux MFS transporter), forward primer, XbaI
NW26	GCGCGGCCGCGTACAGGCTGCAAAGCATCA	end of PP1273 (multidrug efflux MFS transporter), reverse primer, NotI
CmF-AvaI	GCCCCGGGGCTCACTGCCCGCTTTCCA	start of *Cm* marker from pBBR1mcs (6), forward primer, AvaI
Cm_R_MluI	GCACGGCTCGAGATTTTCAGGAGCTAAGGA	end of *Cm* marker from pBBR1mcs (6), reverse primer, MluI
RV165	TGACCAGCTGCGAAGTG	end of *tac* ribosomal binding site, reverse primer, pvuII
KN34	TCATGACGGATTCACCCTTGGCGTCC	start of *Gm* marker from pJNNmcs(t) (26), forward primer, pagI
KN30	CCATGGTGACAATTTACCGAACAACTCC	end of *Gm* marker from pJNNmcs(t) (26), reverse primer, NcoI
KN48	GCGGAATTCATGTATTCTCATGACTTCCCTG	start of PP1271, forward primer, EcoRI
KN49	GCGGCGGCCGCCTAACCGTGAGCATTCGGCG	end of PP1273, reverse primer, NotI

[a] Restriction sites are underlined.

pNagAa promoter, *tac* ribosomal binding site and Cm marker were amplified using primers RV165 and Cm_R_MluI (Table 2) and digested with Kpn2I and XmajI. The resulting fragment was ligated in a Kpn2I and XbaI (compatible with XmajI) digested pBBR1mcs vector, yielding pBNTmcs(Cm^r). The Cm marker was replaced by a gentamicin (Gm) marker. The Gm marker of pJNNmcs (previously known as pTN-1 (26)) was obtained by PCR using primers KN34 and KN30 (Table 2) and cloned into the PagI and NcoI restriction sites of pBNTmcs(Cm^r), yielding pBNTmcs(Gm^r).

For constructing the MFS-transporter expression plasmid pBNTmfs(Gm^r), the multidrug efflux MFS transporter (PP1271-PP1273) was amplified by PCR on genomic DNA of *P. putida* S12 using oligonucleotide primers KN48 and KN49 (Table 2). The resulting fragment was digested with KpnI and NotI and ligated into KpnI-NotI-digested pBNTmcs(Gm^r).

Plasmid pJT'Tmcs was constructed by digesting pJTTmcs (7) with HindIII and BamHI and the resulting fragment was treated with S1 nuclease, according to manufacturer's instructions. The nuclease treated vector was ligated, yielding pJT'Tmcs. For constructing pJT'Tpal, the *pal* gene was obtained from pJTTpal as a KpnI-NotI fragment and purified from agarose gel. The purified fragment was ligated into KpnI-NotI-digested pJT'Tmcs, yielding pJT'Tpal.

Results

General expression features of p-*hydroxybenzoate producing* P. putida *S12palB1*

P. putida S12palB1 was cultured in carbon limited chemostats with either glycerol or glucose as the carbon source. In agreement with previous observations (20), the carbon source had no major effect on product formation: on either substrate comparable concentrations of *p*-hydroxybenzoate and *t*-cinnamate were produced, at a similar product-to-substrate yield (Y_{ps}) (Table 3). The biomass density as well as the biomass-to-substrate yield was slightly higher on glycerol (Table 3; data not shown). As control cultures, wild type *P. putida* S12 variant strains were cultivated in chemostats and harboured either an empty expression plasmid (pJNNmcs(t); (25)), or the Pal/Tal expression plasmid (pJTTpal; (10)) (Table 3). Samples were drawn from the chemostat cultivations for transcriptomics and proteomics analysis. Comparisons were made between the optimized *p*-hydroxybenzoate producing strain *P. putida* S12palB1 and the non-optimized control strains, on either glucose or glycerol as the carbon source. Thus, the responses underlying the improved performance of the platform

Table 3. The biomass and product formation in chemostat cultivations.

strain	carbon source	CDW (g l^{-1})^a	*p*-hydroxybenzoate (mM)^a	*t*-cinnamate (mM)^a	Y_{ps} (Cmol%)^a
P. putida S12 pJNNmcs(t)	glucose	0.65 (±0.02)	0	0	
P. putida S12 pJTTpal	glycerol	0.69 (±0.03)	0	0.022 (±0.005)	
P. putida S12palB1	glucose	0.63 (±0.01)	0.520 (±0.009)	0.050 (±0.004)	6.0 (± 0.1)
P. putida S12palB1	glycerol	0.68 (±0.02)	0.532 (±0.011)	0.052 (±0.009)	6.1 (± 0.1)

[a] Average of three chemostat cultures, errors represent the standard deviation. CDW: cell dry weight; Y_{ps}: *p*-hydroxybenzoate to substrate yield in C-mol *p*-hydroxybenzoate per C-mol substrate (C-mol%).

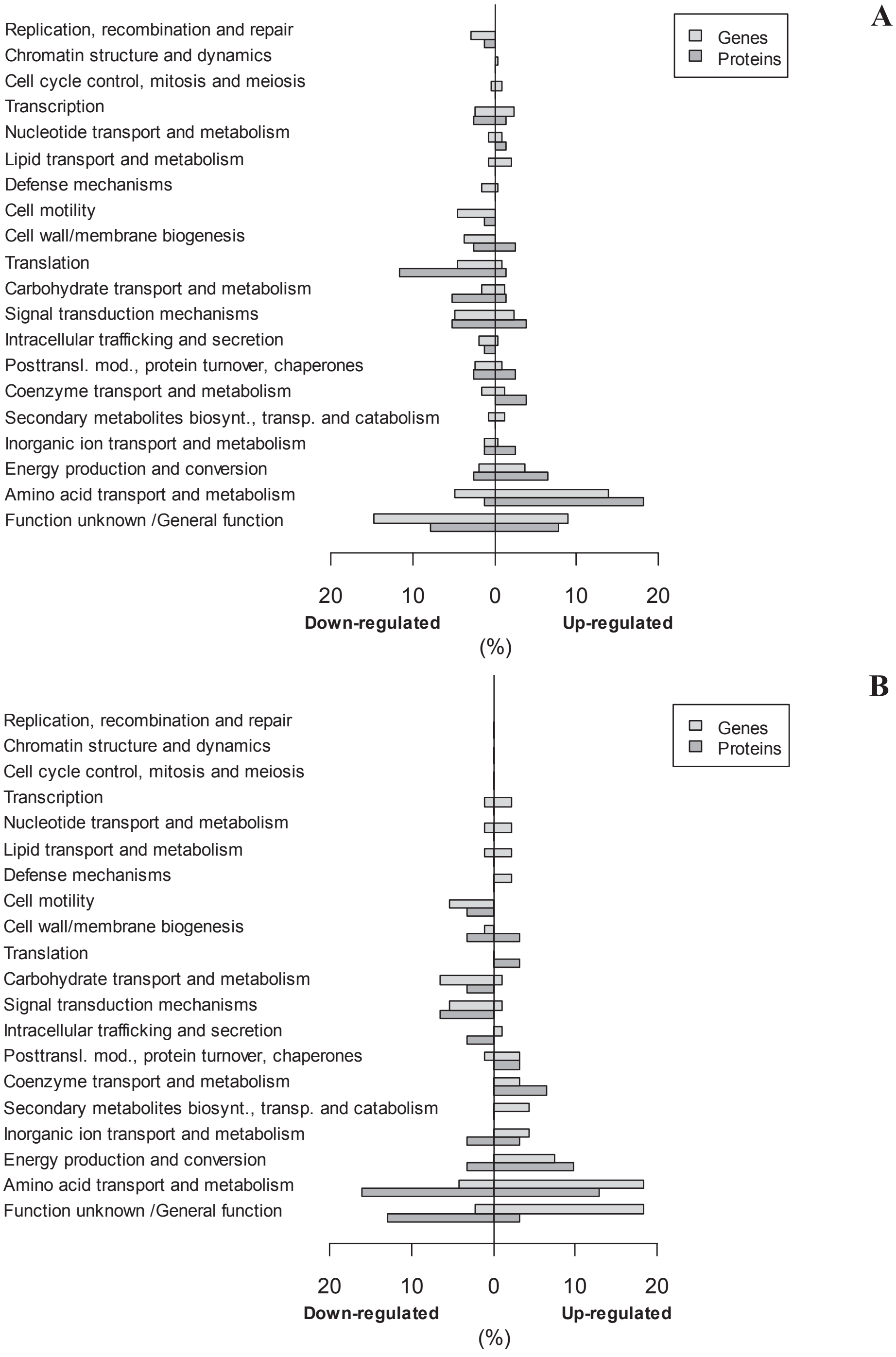

Figure 2. Global response on transcriptome and proteome level between the control strain and P. putida *S12palB1 on glucose (A) and glycerol (B). Percentages of differentially expressed genes (fold changes of at least 2.5) and proteins (fold change of at least 1.5) are given per COG group.*

strain *P. putida* S12_427, as well as the impact of metabolic engineering on strain S12_427 to generate the *p*-hydroxybenzoate producing strain *P. putida* S12palB1 (Fig. 1), could be assessed for either carbon source.

Remarkably, the carbon source employed appeared to have a major effect on global transcript levels in the *p*-hydroxybenzoate producing *P. putida* S12palB1. With glucose as the carbon source, 215 genes were differentially expressed (fold change > 2.5) in *P. putida* S12palB1 compared to the control strain, whereas only 88 genes were differentially expressed on glycerol. Proteome analysis showed a similar trend with 285 differentially expressed spots (fold change >1.5) in glucose-grown *P. putida* S12palB1 compared to the control strain, *vs* 110 spots in the glycerol-grown cultures. From the glucose cultures, 93 out of the 285 spots were analyzed, representing 60 different proteins. Of the 110 differentially expressed spots of the glycerol cultures 39 were analyzed representing 26 unique proteins.

The distribution of the differentially expressed genes and proteins over the different COG categories (based on the NCBI-COG database of *P. putida* KT2440 (http:://www.ncbi.nlm.nih.gov/sutils/coxik.cgi?gi=266)) was very similar for glucose and glycerol-grown *P. putida* S12palB1 (Fig. 2A and B). Nearly all COG groups were represented. About one quarter of the differentially expressed genes and proteins were annotated as having a hypothetical (including unknowns) or general function only. Another 25 % could be assigned to amino acid transport and metabolism. A similar response had been observed previously in the phenol-producing *P. putida* S12TPL3. This was proposed to relate to the selection procedure by which the parent strain *P. putida* S12_427 was obtained, *i.e.*, resistance to fluoroanalogs of the amino acids phenylalanine and tyrosine (26). In addition, the COG groups of transcription and translation were well-represented, indicative of a substantial overall phenotypic difference between the *p*-hydroxybenzoate production strain and the control strain. The slight overrepresentation of the COG groups ‘energy production and conversion’ and ‘signal transduction’ were likely to relate to a solvent stress response, relating to the accumulation of *p*-hydroxybenzoate (Fig. 2; 11, 12)).

Specific expression features of P. putida *S12palB1 relating to p-hydroxybenzoate production*

A number of differentially expressed genes and proteins in *P. putida* S12palB1 could be clearly linked to improved *p*-hydroxybenzoate production. These were classified into three functional groups: 1. aromatic amino acid biosynthesis and *p*-coumarate catabolism (*i.e.*, the synthetic pathway of *p*-hydroxybenzoate biosynthesis), 2. aromatic amino acid catabolism and 3. transport systems (Table 4; Fig. 3). A complete overview of differentially expressed genes and proteins is provided in Verhoef *et al* 2010 Appl Microbiol Biotechnol (Table 1 of the supplemental data).

Aromatic amino acid biosynthesis and *p*-coumarate degradation

In *P. putida* S12palB1, five genes of the tyrosine biosynthetic pathway were upregulated, among which the *aroF-1* gene that was present in an additional copy in this strain (Table 4). Moreover, the tryptophan biosynthetic pathway was found to be upregulated, both at the transcript level (*trpCDGE*) and at the protein level (TrpCDGE as well as TrpA; Table 4). These findings are in good agreement with previous results in phenol producing *P. putida* S12TPL3 (25) as well as with the improved *p*-hydroxybenzoate biosynthesis. The upregulation of the aromatic amino acid biosynthetic genes in general appeared to be somewhat lower on glycerol

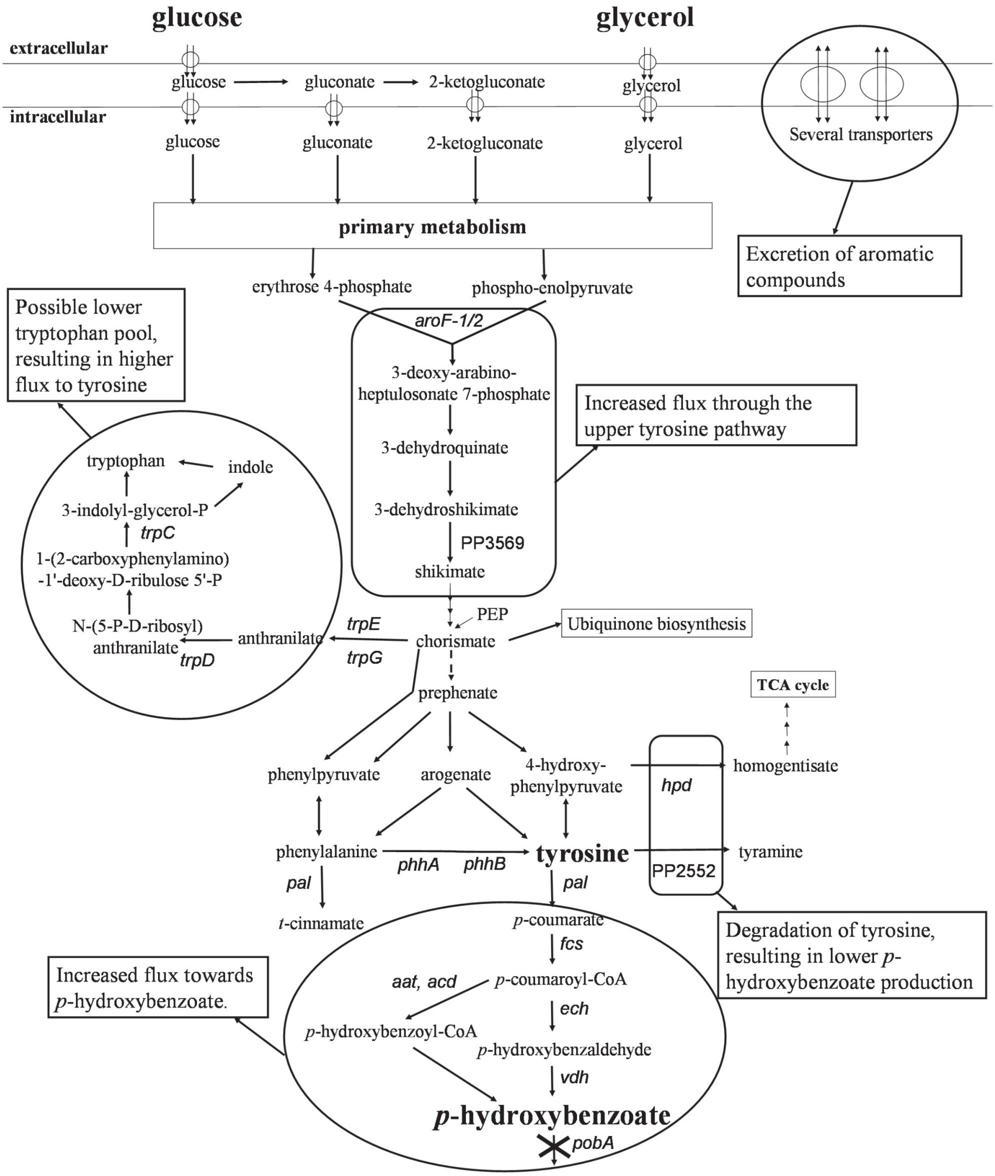

Figure 3. Schematic overview of differentially expressed genes involved in relevant pathways of p-*hydroxybenzoate biosynthesis, as derived from transcriptomics analysis of* P. putida *S12palB1. Gene names are indicated in italic or when absent, indicated by locus tags from* P. putida *KT2440. The text boxes describe the proposed role in the enhanced* p-*hydroxybenzoate production of the differentially expressed genes.*

Table 4. Differentially expressed genes and proteins connected to *p*-hydroxybenzoate production in *P. putida* S12palB1.

Protein description (gene)	KT2440 locus tag	Transcriptome fold changes		Proteome fold changes	
		glycerol[a]	glucose[b]	glycerol[a]	glucose[b]
Aromatic amino acid biosynthesis					
Tryptophan synthase, alpha subunit (*trpA*)	PP0082				1.6
Anthranilate synthase, component I (*trpE*)	PP0417	6.5	9.2	2.7±0.1[c]	13.1±4.2[c]
Anthranilate synthase, component II (*trpG*)	PP0420	4.2	10.3	2.9	2.7
Anthranilate phosphoribosyltransferase (*trpD*)	PP0421	4.4	7.8		
Indole-3-glycerol phosphate synthase (*trpC*)	PP0422	2.6	5.6		4.0
DAHP synthase, class I (*aroF1*)	PP2324	2.5	2.6		
DAHP synthase, class I (*aroF2*)	PP3080	3.1	9.3		
Quinate dehydrogenase (pyrroloquinoline-quinone), putative	PP3569	1.4[d]	2.8		
Phenylalanine-4-hydroxylase (*phhA*)	PP4490	3.3	107.0		
Pterin-4-alpha-carbinolamine dehydratase (*phhB*)	PP4491	3.5	76.3		
***p*-Coumarate catabolism**					
Acyl-CoA dehydrogenase, ferrulic acid biotransformation protein, putative *(acd)*	PP3354	2.5	28.2		
β-ketothiolase *(aat)*	PP3355	1.8[d]	11.9		
Feruloyl-CoA-synthetase (*fcs*)	PP3356	3.6	72.8		
Vanillin dehydrogenase (*vdh*)	PP3357	3.0	37.7		
Enoyl-CoA hydratase aldolase (*ech*)	PP3358	2.5	12.2		
Hydroxycinnamic acid degradation regulator, putative	PP3359	1.9[d]	2.9		
Aromatic catabolism					
Tyrosine decarboxylase, putative	PP2552	0.92[d]	48.0		
4-hydroxyphenylpyruvate dioxygenase (*hpd*)	PP3433	1.5[d]	2.8		
Transport systems					
Amino acid ABC transporter, periplasmic amino acid-binding protein	PP0282				1.6
Aromatic amino acid transporter (*aroP2*)	PP0927	3.0	9.0		
Amino acid ABC transporter ATP-binding protein	PP1068				2.5
Amino acid ABC transporter, periplasmic amino acid-binding protein	PP1071			0.64±0.01[c]	
Branched-chain amino acid ABC transporter, ATP-binding protein (*braG*)	PP1137				2.0

Branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein (*braC*)	PP1141				2.1±0.1[c]
Multidrug efflux MFS transporter, putative	PP1271	3.8	7.6		
Multidrug efflux MFS membrane fusion protein	PP1272	13.7	17.4		
Multidrug efflux MFS outer membrane protein	PP1273	4.8	10.1		
General amino acid ABC transporter, periplasmic binding protein (*aapJ*)	PP1297	0.48[d]	3.4	0.57±0.06[c]	5.1±0.6[c]
General amino acid ABC transporter, permease protein (*aapQ*)	PP1298	0.51[d]	4.6		
General amino acid ABC transporter, permease protein (*aapM*)	PP1299	0.70[d]	2.9		
General amino acid ABC transporter, ATP-binding protein (*aapP*)	PP1300	0.56[d]	3.0	0.61	2.6
Amino acid transporter, AAT family	PP3727	2.5	4.2		
Aromatic amino acid transporter (*aroP1*)	PP4495	4.0	14.1		
Branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein	PP4867			0.65	2.4±0.1[c]

[a] Fold change in expression level of *P. putida* S12palB1 compared to *P. putida* S12 pJTTpal on glycerol as substrate; values below 1 represent down regulation and values above 1 represent upregulation in *P. putida* S12palB1

[b] Fold change in expression level of *P. putida* S12palB1 compared to *P. putida* S12 pJNNmcs(t) on glucose as substrate; values below 1 represent down regulation and values above 1 represent upregulation in *P. putida* S12palB1

[c] Average of all spots per protein, errors represent the maximum deviation from the mean. See supplemental data for values per spot.

[d] Below threshold

compared to glucose (by a factor 1.5 – 3). An exception was the dramatic effect observed for *phhAB*, the upregulation of which was lower by a factor 20-30 on glycerol-grown *P. putida* S12palB1 compared to glucose-grown cells.
In addition to the aromatic amino acid biosynthetic genes, all genes involved in the conversion of *p*-coumarate into *p*-hydroxybenzoate (5, 8, 19) were upregulated compared to the control strains (Table 4). Again, the level of upregulation of these genes was apparently carbon-source dependent, being lower by an order of magnitude in the glycerol-grown cells compared to the glucose-grown cultures.

Tyrosine degradation pathways

In addition to the tyrosine biosynthetic genes, genes involved in the degradation of tyrosine were upregulated (Table 4, Fig. 3), although exclusively in glucose-grown cultures. The tyrosine decarboxylase-encoding gene PP2552 was upregulated 48-fold in glucose-grown *P. putida* S12palB1. However, no accumulation of tyramine was observed. Considering that tyramine is a dead-end product, the contribution of tyrosine decarboxylase to tyrosine catabolism appeared to be limited. As suggested earlier for phenol producing *P. putida* S12TPL3 (25), degradation of tyrosine is more likely to occur via the homogentisate pathway of which the upregulated *hpd* gene encodes the first enzyme, *i.e.*, 4-hydroxyphenylpyruvate dioxygenase.

Transport systems

The extracellular accumulation of *p*-hydroxybenzoate in cultures of *P. putida* S12palB1 is likely to involve cellular transport systems. This is supported by the upregulation of numerous transport systems in *P. putida* S12palB1 compared to the control strains. Among these were a multidrug efflux MFS transporter (PP1271 – PP1273) and a wide range of (aromatic) amino acid transporters (Table 4). The nature of the carbon source employed apparently also affected the expression of these transporters. The upregulation of the MFS transporter genes PP1271-1273 showed little carbon-substrate dependency, being slightly lower on glycerol than on glucose. For the amino acid transporter-encoding genes, much larger upregulation was observed depending on the carbon source used. Remarkably, at the protein level even downregulation of some amino acid transporters was observed in glycerol-grown *P. putida* S12palB1 compared to the control strain, as opposed to the glucose-grown cultures (Table 4).

Improvement of p-*hydroxybenzoate production by targeted deletion of the* hpd *gene*

We previously demonstrated that the addition of tyrosine to chemostat-cultivated *P. putida* S12palB1 resulted in improved *p*-hydroxybenzoate titers, indicating that product formation was limited by the availability of tyrosine. However, part of the added tyrosine was consumed to generate biomass indicating that *p*-hydroxybenzoate production competed for tyrosine with other pathways (20). The upregulation of genes involved in tyrosine degradation as observed in the present study is in agreement with these observations. As argued above, tyrosine is probably mainly degraded via the homogentisate pathway. Therefore, the *hpd* gene was disrupted in *P. putida* S12palB1 to improve tyrosine availability.
The resulting strain, *P. putida* S12palB2, showed a marked improvement of *p*-hydroxybenzoate production characteristics in shake flask cultures with glucose as the carbon source. Product titers increased from 1.8 to 2.3 mM compared to strain S12palB1, at a similar final biomass

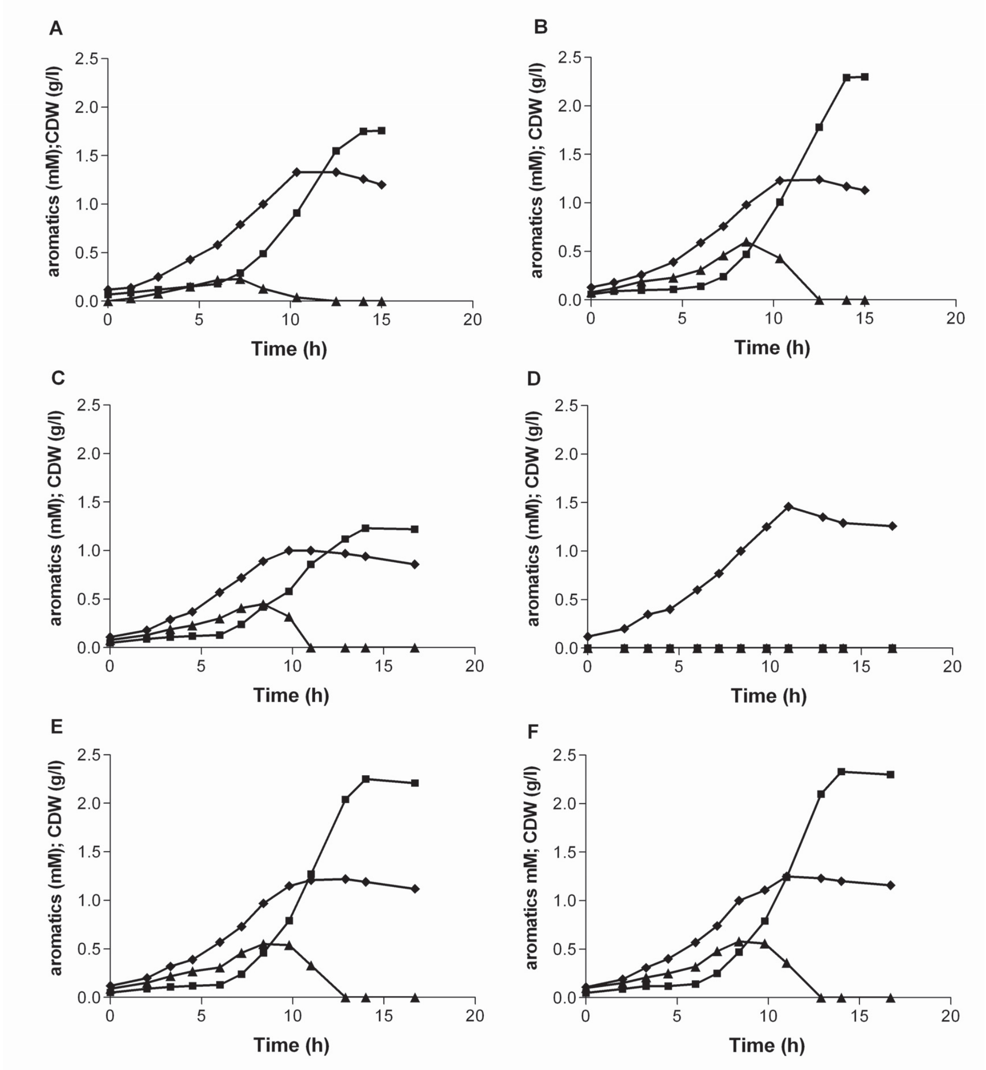

Figure 4. Growth and production of p-*hydroxybenzoate of* P. putida *S12palB1 (A),* P. putida *S12palB2 (B),* P. putida *S12palB3 (C),* P. putida *S12B3 (D),* P. putida *S12pal_mfsB3 (E) and* P. putida *S12pal_mfsB2 (F) in mineral medium with 20 mM glucose as carbon source in shake flask cultures.* p-*hydroxybenzoate (■),* p-*coumarate (▲) and CDW (cell dry weight) (♦). The data are averages of triplicate experiments. The maximum variation between the triplicates was less than 10 %.*

content (Fig. 4A, B). The product-to-substrate yield (Y_{ps}) improved from 10.5 C-mol % for strain S12palB1 to 13.4 C-mol % for stain S12palB2, *i.e.*, a relative yield improvement of 22 %. Also the productivity showed considerable improvement: the overall specific production rate q_p increased from 1.6 to 2.3 μmol *p*-hydroxybenzoate (g CDW min)$^{-1}$, while the maximum specific production rate ($q_{p,max}$) increased from 3.8 to 4.4 μmol *p*-hydroxybenzoate (g CDW min)$^{-1}$. Maximal q_p was achieved between 10 and 12 h of cultivation, in line with the observed transient accumulation of *p*-coumarate (Fig. 4A, B). The latter is likely to relate to transient glucose repression of the *fcs* gene, which immediately affected the *p*-coumarate pool (Fig. 3). Interestingly, when *P. putida* S12palB2 was cultured on glycerol, *p*-hydroxybenzoate production was also improved. The product-to-substrate yield showed a relative increase of 21 %, even though *hpd* was only slightly upregulated (below threshold of 2.5-fold) in glycerol-grown *P. putida* S12palB1. Transient accumulation of *p*-coumarate did not occur on glycerol since *fcs* was not repressed (unpublished data). Thus, disrupting the *hpd* gene clearly had a stimulating effect on *p*-hydroxybenzoate production.

Export of p-*hydroxybenzoate in* P. putida *S12palB1*

Transcriptome and proteome analysis in the present study showed extensive differential expression of cellular transport systems in *P. putida* S12palB1. Among the transporters identified, the multidrug efflux MFS transporter (*P. putida* KT2440 locus tags PP1271 - PP1273) was upregulated on both glucose and glycerol, with only minor variation between the two carbon substrates. Since *p*-hydroxybenzoate accumulation was comparable in glucose and glycerol-grown cultures, the MFS transporter was considered as a putative *p*-hydroxybenzoate exporter in *P. putida* S12. To assess the role of this transporter in *p*-hydroxybenzoate transport, all three ORFs were deleted in *P. putida* S12palB2, yielding strain *P. putida* S12palB3. The inactivation of the MFS transporter resulted in slightly impaired growth and significantly lower *p*-hydroxybenzoate titers, although production was not completely eliminated (Fig. 4C). When the Pal/Tal expression plasmid pJTTpal was removed from *P. putida* S12palB3, resulting in the non-producing strain *P. putida* S12B3, normal growth was restored (Fig. 4D). Thus, the growth impairment observed in *P. putida* S12palB3 must be attributed to a combined effect of the transporter knock-out and intracellular *p*-hydroxybenzoate production.

Complementation of the MFS transporter in *P. putida* S12palB3 restored normal growth as well as production of *p*-hydroxybenzoate (Fig. 4E). These results suggest that the MFS transporter PP1271-1273 plays an important role in *p*-hydroxybenzoate export. Overexpression of the MFS transporter in *P. putida* S12palB2 did not result in improved *p*-hydroxybenzoate production (Fig. 4F). Since the complementation experiment indicated (Fig. 4E) that the MFS transporter was functionally expressed the MFS transporter clearly is not a limiting factor for *p*-hydroxybenzoate production.

Discussion

In the present study, the changes in transcript and protein levels underlying and affecting efficient *p*-hydroxybenzoate biosynthesis by *P. putida* S12palB1 have been studied. Many of the –extensive- general expression changes were found to relate to system-wide alterations originating from the optimization of the original platform strain (20, 25, 26). In addition, also *p*-hydroxybenzoate production appeared to have a substantial general impact. Part of the responses could be attributed to *p*-hydroxybenzoate stress, whereas others may be related to the ‘short-circuiting’ of an anabolic pathway (aromatic amino acid biosynthesis) and a catabolic pathway (*p*-coumarate degradation).

The pathways directly involved in *p*-hydroxybenzoate formation were considerably upregulated in *P. putida* S12palB1, in line with the elevated metabolic flux towards the product. These responses were comparable to those observed in *P. putida* S12TPL3 (25). The upregulation of the *p*-coumarate pathway is obviously unique for strain S12palB1 as this pathway requires the presence of *p*-coumaroyl-CoA for induction (2). By contrast, no responses of the protocatechuate pathway were observed which is explained by the disruption of the first gene of this pathway, *pobA*, in *P. putida* S12palB1. The homogentisate pathway was shown to account for substantial loss of tyrosine, thus compromising *p*-hydroxybenzoate synthesis. Considerable improvement of both yield and productivity was achieved by targeted inactivation of *hpd*, the first gene of the homogentisate pathway.

Remarkably, the nature of the carbon source employed exerted major effects in *P. putida* S12palB1. Effects were observed both at the global expression level and specifically for genes and proteins that are directly associated with *p*-hydroxybenzoate biosynthesis and production. The effect was most prominent for the genes of the *p*-coumarate degradation pathway and the genes encoding phenylalanine hydroxylase, *phhA* and *phhB*. No such differences were observed between the control cultures on glucose and glycerol. Therefore, the carbon source-dependency of the expression of these genes must be considered a characteristic of *P. putida* S12palB1. Nonetheless, the transcript levels in glycerol-grown *P. putida* S12palB1 apparently sufficed to accommodate efficient *p*-hydroxybenzoate biosynthesis, since similar amounts were produced in glucose-grown and glycerol-grown cultures. Moreover, none of the upregulated '*p*-hydroxybenzoate biosynthetic' gene products were detected in the proteomics analysis, which supported the notion that different transcript levels do not necessarily affect actual protein synthesis. It cannot be excluded, however, that the bottleneck for *p*-hydroxybenzoate production on either substrate is located upstream of the tyrosine biosynthetic pathway, *e.g.*, at the level of the key aromatic precursors phosphoenolpyruvate and erythrose-4-phosphate.

In addition to metabolic genes, various transporter systems were differentially expressed in *P. putida* S12palB1. Although the multidrug efflux MFS transporter PP1271-PP1273 showed no similarity to known *p*-hydroxybenzoate transporters such as AaeAB of *Escherichia coli* (18), it was shown to be involved in *p*-hydroxybenzoate export. Overexpression did not improve production, however, suggesting that the innate transport capacity was sufficient to cope with the amount of *p*-hydroxybenzoate generated. The MFS transporter was furthermore not essential, and unlikely to be specific, for *p*-hydroxybenzoate export, since it was also upregulated in response to phenol (unpublished data), toluene (22) and urea (in *P. putida* KT2440; (14)).

In addition to contributing to the general understanding of the genetic background underlying the improved *p*-hydroxybenzoate production characteristics of *P. putida* S12palB1, the transcriptomics and proteomics results provided leads that resulted in further strain improvement. Moreover, the fact that the available carbon source directly impacts the expression of key *p*-hydroxybenzoate biosynthetic pathways indicates that the *p*-hydroxybenzoate production capacity has not been exploited to its full potential. The precise nature of this upstream bottleneck preventing further yield improvement is currently under investigation.

Acknowledgements

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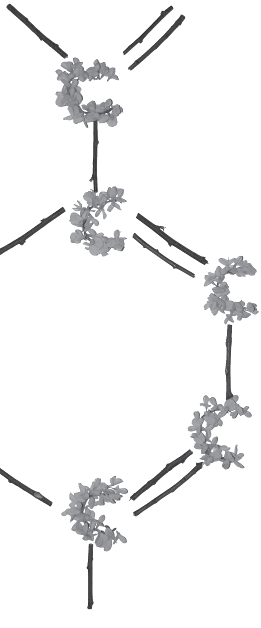
Chapter 4

Improved aromatics production by engineered *Pseudomonas putida* S12 using a mixed substrate-feeding strategy



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Meijnen JP[*], Verhoef S [*], Briedjlal AA, de Winde JH and Ruijssenaars HJ Improved aromatics production by engineered *Pseudomonas putida* S12 using a mixed substrate-feeding strategy.

[*] Both authors contributed equally to this work

Abstract

The key precursors for aromatics production by engineered *P. putida* S12 are phospho-enol pyruvate (PEP) and the pentose-phosphate (PP) pathway intermediate erythrose-4-phosphate (E4P). Since the PP pathway fluxes are typically low in Pseudomonads, E4P availability may be improved by co-feeding pentoses to stimulate the PP pathway. For this purpose, a xylose catabolic pathway was engineered into the *p*-hydroxybenzoate producing strain *P. putida* S12palB2, followed by evolutionary selection for an improved xylose utilization phenotype. Aromatics production was considerably enhanced by co-feeding xylose to glucose or glycerol, resulting in up to 2-fold improved yield. Also without xylose, *p*-hydroxybenzoate production was improved, apparently due to the evolutionary selection that yielded an elevated PP pathway-activity phenotype. The yield improvements were most pronounced with glycerol as the primary substrate. This probably related to the availability of glyceraldehyde-3-phosphate (G3P) which is the precursor for PEP but also feeds the PP pathway. Since glucose is metabolized via the Entner-Doudoroff pathway, the theoretical G3P availability from glucose is half of that on glycerol. Furthermore, when co-fed with glucose, xylose was utilized incompletely. Hence, we inferred that xylose import was PEP dependent. Although this would present an additional drain on PEP, the overall effect of xylose-cofeeding on aromatics biosynthesis was positive.

Introduction

Pseudomonas putida S12 is a solvent-tolerant bacterium that has been developed as a platform host for the production of substituted aromatic compounds (12, 20, 21, 23). These compounds are derived from the aromatic amino acid L-tyrosine, the key precursors of which are phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) (Fig 1). Increasing the availability of these intermediates can considerably enhance the production of aromatic compounds, as was recently demonstrated in *Escherichia coli* (8).

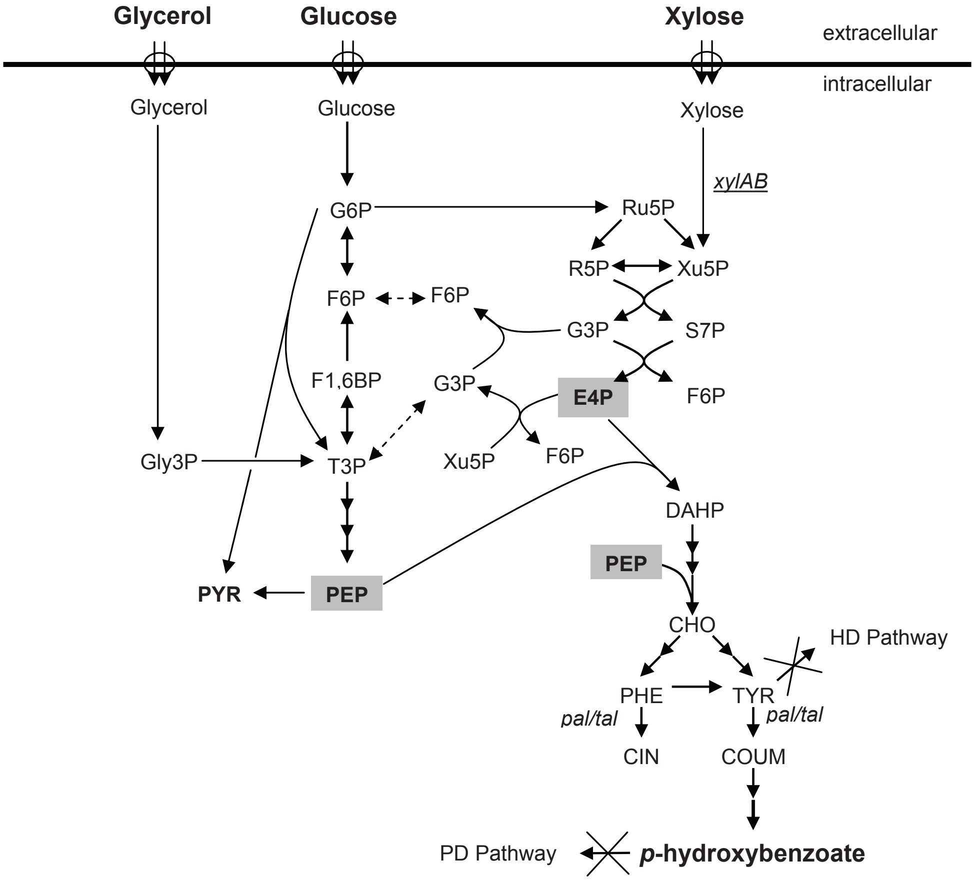


Figure 1. Schematic representation of the biosynthetic pathways for p-*hydroxybenzoate production from glycerol, glucose and xylose. The scheme shows only the relevant routes. Heterologous genes are indicated in italic and underlined. Xylose isomerase (*xylA*); xylulokinase (*xylB*); phenylalanine/tyrosine 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 (X5P); ribose-5-phosphate (R5P); glyceraldehyde-3-phosphate (G3P); 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).*

PEP is produced in the lower glycolysis from glyceraldehyde-3-phosphate (G3P), whereas E4P is derived from the pentose phosphate (PP) pathway. The activity of the PP pathway has been shown to be very low in *P. putida* strains when grown on glucose (2, 4, 22) or on glycerol (manuscript in preparation). Since these substrates are commonly used for aromatics production (11, 12, 20, 21, 23), the E4P availability is a probable bottleneck under these conditions. Therefore, it was anticipated that aromatics production could be improved by enhancing the PP pathway fluxes, *e.g.*, by co-feeding pentose sugars (5).

P. putida S12, however, is not able to utilize pentoses as carbon source. Thus, to enable increased E4P availability via pentose co-feeding, we introduced a phosphorylative xylose utilization pathway in the *p*-hydroxybenzoate producing strain *P. putida* S12palB2 (19) analogous to the previous approach of Meijnen *et al*. (9). The effect of xylose co-feeding on *p*-hydroxybenzoate production was assessed in chemostat experiments using glucose as the primary carbon source. In terms of PEP availability, however, glucose was expected to be a sub-optimal substrate. *P. putida* utilizes glucose exclusively via the Entner-Doudoroff (ED) pathway, yielding equimolar amounts of G3P and pyruvate. Therefore, maximally 50 % of the glucose can be converted into PEP, assuming that pyruvate dikinase (PEP synthase) is active exclusively under gluconeogenic conditions (17). Glycerol, on the other hand, is metabolized completely via G3P (Fig. 1) and can theoretically be fully converted into PEP, resulting in minimal 'losses' of carbon for *p*-hydroxybenzoate production. Co-feeding of xylose and glycerol was therefore expected to improve both PEP and E4P availability and, thus, *p*-hydroxybenzoate 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) (15) and a phosphate buffered minimal medium (MM; (19)). In minimal media, 12 mM of xylose (MMX), 10 mM of glucose (MMG) or 20 mM of glycerol (MMGly) was used as the sole carbon source, unless stated otherwise. Antibiotics were added as required to the media to the following final concentrations: gentamicin, 10 µg ml^{-1} (MM) or 25 µg ml^{-1} (LB); tetracycline, 10 µg ml^{-1} (*E. coli*), 40 µg/ml^{-1} (*P. putida* S12 in MM) or 60 µg ml^{-1} (*P. putida* S12 in LB). In view of the photo sensitivity of tetracycline, amber bottles and light tight fermentors were employed for culturing. The expression of the introduced copy of *aroF-1* as well as the expression of the *xylAB_FGH* genes on the pJNTxylAB_FGH vector was induced by addition of 0.1 mM of sodium salicylate.

Batch experiments were performed in boston bottles containing 20 ml of minimal medium in a horizontally shaking incubator at 30 °C. Cultures were inoculated to a starting optical density at 600 nm (OD_{600}) of 0.1 with cells precultured on MM with xylose, glucose or glycerol, depending on the carbon source to be used.

Carbon-limited chemostat cultivations were performed as described previously (20) in 1-l fermentors with a BioFlo110 controller (New Brunswick Scientific) using MM containing different mixtures of glycerol and xylose or glucose and xylose (total carbon (C) concentration: 60 mM), 10 µg l^{-1} gentamicin, 40 µg l^{-1} tetracycline and 0.1 mM sodium salicylate. Chemostats were inoculated with a 35-ml inoculum of a late-log phase preculture

Table 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics [a]	Source
Strains		
P. putida S12B2	*pobA* and *hpd* knockout strain with an enhanced flux towards tyrosine	(19)
P. putida S12palB2	*P. putida* S12B2 containing plasmid pJT'Tpal	(19)
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* S12xylB6 containing plasmid pJNTxylAB_FGH	This study
P. putida S12xylB7	*P. putida* S12xylB6 evolved to efficient xylose utilizer	This study
P. putida S12pal_xylB7	*P. putida* S12xylB7 containing plasmid pBT'Tpal	This study
Escherichia coli DH5α	*supE*44 Δ*lac*U169 (φ80 *lac*ZΔM15) *hsdR*17 *rec*A1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Invitrogen
Plasmids		
pJT'Tmcs	Ap^r, Gm^r, basic expression vector for P*tac* and *tac* RBS controlled expression	(19)
pJNTmcs(t)	Ap^r, Gm^r; basic expression vector containing the salicylate-inducable promoter *nagR-nagAa* and *tac* RBS	(9)
pJNTxylAB_FGH	pJNTmcs(t) containing the *xylAB_FGH* genes from *E. coli* DH5α	(9)
pBT'Tmcs	Tc^r; basic expression vector for P*tac* and *tac* RBS controlled expression	This study
pBT'Tpal	pBT'Tmcs containing the *pal* gene from *R. toruloides*	This study
pJQ200SK	P15A *ori sacB* RP4 Gmr(pBluescriptSK); suicide vector	(14)
pJQgcd::tetA_loxP	Gm^r Tc^r, pJQ200SK containing a *loxP-tetA-loxP* interrupted copy of the *gcd* gene	This study

[a] Ap^r, Gm^r and Tc^r, ampicillin, gentamicin and tetracycline resistance respectively.

on MMX. For chemostat cultivations on xylose as sole carbon source, the dilution rate (D) was set at 0.05 h^{-1} since wash-out occurred at higher dilution rates. For glycerol-xylose mixtures, the D was initially set to 0.05 h^{-1}. When steady state was reached, the cultures were sampled and D was increased to 0.1 h^{-1}. Upon reaching steady state at D = 0.1 h^{-1}, the cultures were sampled again. For glucose-xylose mixtures, the D was set at 0.1 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 5 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. *p*-Hydroxybenzoate was analyzed by HPLC (Agilent 1100 system) using a Zorbax 3.5 µm SB-C18 column (4.6 x 50mm) and a diode-array detector set at 254 nm. As the eluent, a linear gradient of acetonitrile in KH_2PO_4-buffer (50 mM, pH 2, 1 % acetonitrile) was used, increasing from 0-25 % 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 (9, 19).

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 pBT'Tmcs (Tc^r) the *tac* expression cassette and chloramphenicol (Cm) marker of pJT'Tmcs (19) were amplified by PCR using primers 1 and 2 (Table 2). The resulting PCR product was digested with *Kpn*2I and *Xma*JI (restriction sites present in the amplified fragment) and consequently ligated in a *Kpn*2I - *Xba*I (compatible with *Xma*JI) digested pBBR1mcs vector, yielding pBT'Tmcs (Cm^r). 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 (7) and cloned into the *Pag*I and *Nco*I restriction sites of pBT'Tmcs (Cm^r), yielding pBT'Tmcs (Tc^r).

For constructing pBT'Tpal, the *pal* gene from pJTTpal (20) was obtained as a *Kpn*I-*Not*I fragment and purified from agarose gel. The purified fragment was ligated into *KpnI-NotI*-digested pBT'Tmcs, yielding pBT'Tpal.

Expression vector pJNTxylAB_FGH was constructed by cloning *xylAB* and *xylFGH* in vector pJNTmcs (9). The genes were amplified by PCR using genomic DNA from *E. coli* DH5α as the template and oligonucleotide primers 5-8 (Table 2). The resulting fragments were ligated into vector pJNTmcs using the restriction sites *Kpn*I and *Not*I for *xylAB*, and *Nhe*I and *Sfi*I for *xylFGH*. The resulting plasmid was designated pJNTxylAB_FGH.

Targeted gene disruption

The *gcd* knock-out mutant of *P. putida* S12B2 (19) was constructed in analogy to the *gcd* knock-out mutant of wild-type *P. putida* S12 (9). The gene replacement plasmid for the *gcd* gene, pJQgcd::tetAloxP was constructed from the suicide vector pJQ200SK (14) based on the pJQgcd::Kana vector (9). The *loxP-kanaR-loxP* fragment, encoding kanamycin resistance in vector pJQgcd::Kana was replaced by the *loxP-tetA-loxP* fragment (16, 18), coding for tetracycline resistance, using *Xba*I.

Table 2. Oligonucleotide primers used in this study.

Primer	Sequence [a]	Characteristics
Primer 1	TGACCAGCTGCGAAAGTG	Replicon of pJT'Tmcs ,*Pvu*II
Primer 2	GCGACGCGTGCGCACATTTCCCCGAAAAGTGC	End of Cm^R marker, *Mlu*I
Primer 3	CGCGAATTCTCATGTTTGACAGCTTATCATCG	Start of Tc^R marker, *Eco*RI
Primer 4	CGCGAATTCCCTCAGGTCGAGGTGGCC	End of Tc^R marker, *Eco*RI
Primer 5	GCGGCGGGTACCATGGAGTTCAATATGCAAGC	Start of *xylA* from *E. coli*, *Kpn*I
Primer 6	GCGGCGGCGGCCGCTTACGCCATTAATGGCAG	End of *xylB* from *E. coli*, *Not*I
Primer 7	GCGGCGGCTAGCATGAAAATAAAGAACATTCTACTC	Start of *xylF* from *E. coli*, *Nhe*I
Primer 8	GCGGCGGGCCTAGGCGGCCTCAAGAACGGCGTTTGGTTGCGGA	End of *xylH* from *E. coli*, *Sfi*I

[a] The restriction sites used for cloning are underlined.

Results

Construction of a xylose-utilizing p*-hydroxybenzoate-producing strain*

Prior to engineering for xylose utilization, the *p*-hydroxybenzoate producing strain *P. putida* S12palB2 (Table 1; (19)) was cured from the *pal/tal* expression plasmid pJT'Tpal. Subsequently, the *gcd* gene encoding glucose dehydrogenase was disrupted in order to prevent oxidation of xylose to xylonate, which is essential for efficient phosphorylative xylose utilization (10). The *xylAB_FGH* genes from *E. coli* DH5α (encoding xylose isomerase, xylulokinase and a high-affinity xylose transporter) were introduced and growth of the resulting strain *P. putida* S12xylB6 was assessed in minimal medium with xylose. Slow growth was observed after 16 days of incubation. In order to optimize the growth performance, evolutionary selection was performed as described previously (9). After four transfers *P. putida* S12xylB7 was obtained that exhibited a maximum growth rate of 0.34 h^{-1} on xylose and a biomass-to-substrate yield (Y_{xs}) of 51 Cmol %. Surprisingly, it took 10-fold less transfers for *P. putida* S12xylB7 to achieve growth characteristics similar to those of the previously evolved *P. putida* S12xylAB2 (9). The low number of transfers could partly be explained by the targeted disruption of *gcd* in *P. putida* S12xylB6, since more than ten transfers were required for strain S12xylAB2 to acquire a *gcd* negative phenotype. In addition, the intrinsic PP pathway activity of the parent strain *P. putida* S12palB2 is likely to be elevated in view of the improved metabolic flux towards tyrosine (22).

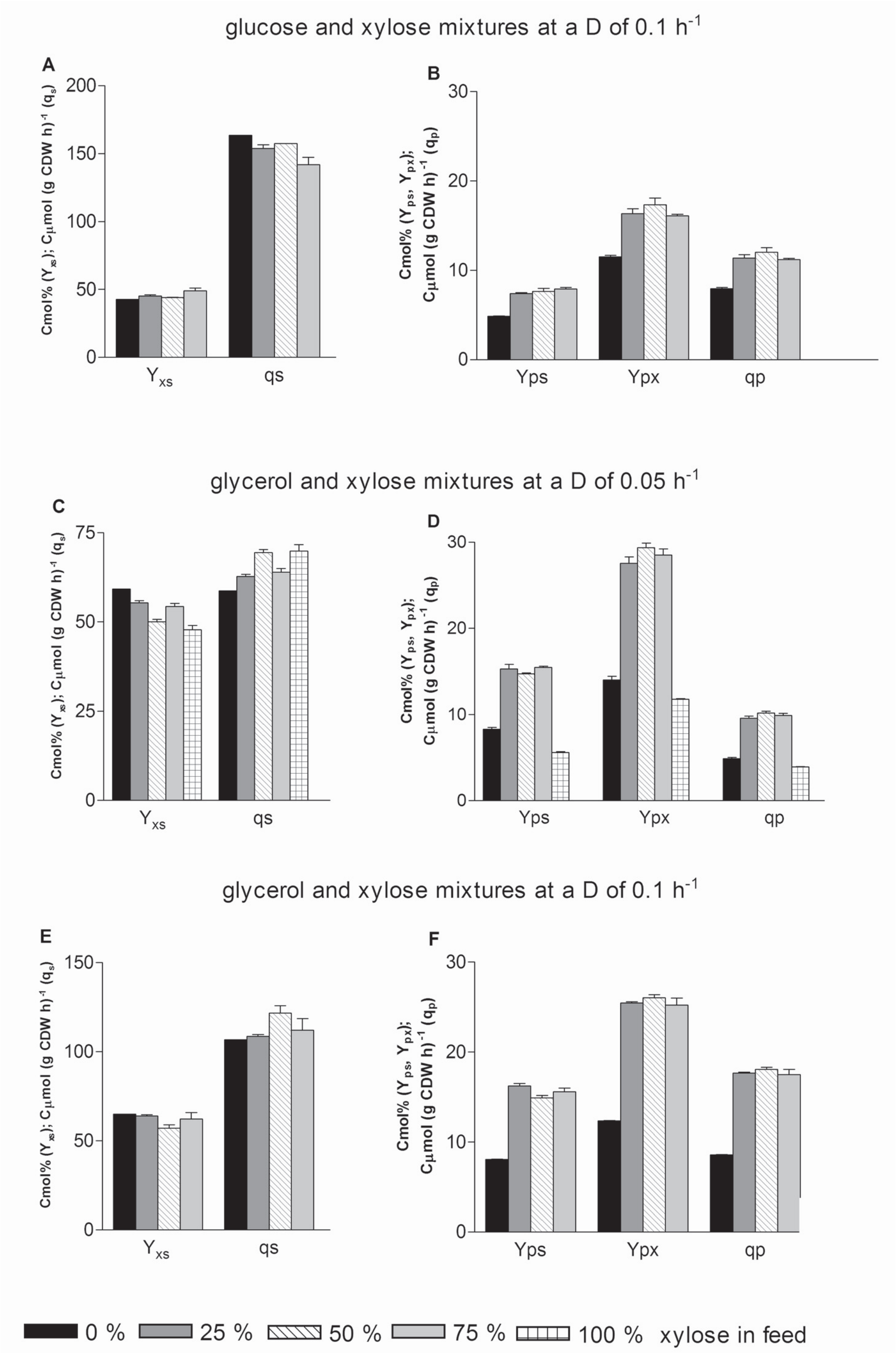
Production of *p*-hydroxybenzoate was restored in *P. putida* S12xylB7 by introducing the phenylalanine/tyrosine ammonia lyase (*pal/tal*) expression plasmid pBT'Tpal, yielding strain *P. putida* S12pal_xylB7. In batch cultivations with xylose as single carbon source, *P. putida* S12pal_xylB7 produced *p*-hydroxybenzoate at a product-to-substrate yield (Y_{ps}) of 12.4 Cmol % (Table 3). Remarkably, the introduction of *pal/tal* resulted in a three-fold reduction of the maximum growth rate on xylose (not shown).

The subsequent evolutionary selection procedure, aiming at an improved growth phenotype on xylose, apparently also resulted in improved *p*-hydroxybenzoate production on other substrates. The product-to-substrate yield on glucose increased from 14.2 Cmol % for *P. putida* S12palB6 (Table 1) to 17.4 Cmol % for *P. putida* S12pal_xylB7. On glycerol, Y_{ps} improved from 15.4 to 19.3 Cmol % (data not shown versus Table 3).

Effect of xylose co-feeding on p*-hydroxybenzoate production*

When the engineered strain *P. putida* S12xylAB2 was grown in batch cultivations on mixtures of glucose and xylose, a diauxic shift was observed with glucose as the preferred carbon source (9). Obviously, the occurrence of diauxy would invalidate the concept of co-feeding

Figure 2. Growth and p*-hydroxybenzoate production of* P. putida *S12pal_xylB7 in chemostat cultivations on various mixtures of carbon sources at various dilution rates (D). The biomass-to-substrate yield (Y_{xs}), product-to-substrate yield (Y_{ps}) and product-to-biomass yield (Y_{px}) expressed as in Cmol biomass or product per Cmol substrate or biomass. The specific carbon uptake rate (q_s) and specific* p*-hydroxybenzoate production rate (q_p) were given in Cµmol substrate or product per g cell dry weight (CDW) per h.*


glucose and xylose mixtures at a D of 0.1 h-1
A
Cmol% (Yxs); Cμmol (g CDW h)-1 (qs)
200
150
100
50
0
Yxs
qs
B
Cmol% (Yps, Ypx); Cμmol (g CDW h)-1 (qp)
30
20
10
0
Yps
Ypx
qp
glycerol and xylose mixtures at a D of 0.05 h-1
C
Cmol% (Yxs); Cμmol (g CDW h)-1 (qs)
75
50
25
0
Yxs
qs
D
Cmol% (Yps, Ypx); Cμmol (g CDW h)-1 (qp)
30
20
10
0
Yps
Ypx
qp
glycerol and xylose mixtures at a D of 0.1 h-1
E
Cmol% (Yxs); Cμmol (g CDW h)-1 (qs)
150
100
50
0
Yxs
qs
F
Cmol% (Yps, Ypx); Cμmol (g CDW h)-1 (qp)
30
20
10
0
Yps
Ypx
qp
0 %
25 %
50 %
75 %
100 %
xylose in feed

two substrates simultaneously to different pathways in order to improve the availability of two key precursors. Therefore, chemostat cultivations were performed with mixed-substrate feeds in which glucose or glycerol was the limiting nutrient. Varying amounts of xylose were used (0 to 75 % of 60 mM total carbon; Fig. 2), replacing the primary carbon source. In agreement with the considerably reduced growth rate on xylose, *P. putida* S12pal_xylB7 washed out above a dilution rate of 0.05 h^{-1} when xylose was used as the sole carbon source. Therefore, one series of chemostats (with glycerol-xylose feed) was operated at a dilution rate of both 0.05 and 0.1 h^{-1}. Figures 2 C-F show that both biomass and product yields were not affected by the dilution rate, whereas the specific production and substrate uptake rates were proportional to the dilution rate, as expected. In order to attain maximum productivity, the co-feeding experiments with glucose-xylose mixtures were performed at a dilution rate of 0.1 h^{-1} only.

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 rate of 8.0 µmol C (g CDW)$^{-1}$ h^{-1} (Fig. 2B). When xylose was co-fed with glucose, the production of *p*-hydroxybenzoate improved significantly. The product-to-substrate yield reached a maximum of 7.9 Cmol % and the specific production rate increased by a factor of 1.5 to 12.0 µmol C (g CDW)$^{-1}$ h^{-1} (Fig 2B). Surprisingly, the amount of xylose being co-fed did not significantly affect Y_{ps} and q_p within the range tested (Fig. 2B). On xylose as a single carbon source, the product-to-substrate yield was considerably lower (5.6 Cmol % (Fig. 2C)), although still slightly improved compared to the glucose-fed chemostats (Table 3). These results clearly demonstrated that simultaneous utilization of glucose and xylose was beneficial for *p*-hydroxybenzoate production.

The biomass-to-substrate yield slightly increased with increasing xylose concentrations in the glucose-xylose feed (Fig. 2A). This can be attributed to the higher biomass yield on xylose compared to glucose (Y_{xs} of 47.7 *vs.* 42.5 Cmol %, Fig. 2 C and A).

Production of p-*hydroxybenzoate from mixtures of glycerol and xylose*

With glycerol as the sole carbon source in chemostat cultures, *p*-hydroxybenzoate production by *P. putida* S12pal_xylB7 was more efficient than with glucose or xylose. At 8.5 Cmol %, the Y_{ps} was 1.7-fold higher for glycerol than for glucose. This phenomenon had not been observed in the non-evolved *p*-hydroxybenzoate production strain *P. putida* S12palB1 (19), suggesting a specific benefit of glycerol for aromatics production by *P. putida* S12pal_xylB7.

In addition to the product-to-substrate yield, also the biomass-on-substrate yield was improved on glycerol. Apparently, the overall carbon efficiency was improved on glycerol compared to glucose, which implied that more carbon was directed towards biomass and aromatic product and less carbon was turned into CO_2. The improved biomass yield largely cancelled out the effect of the improved product yield on the specific *p*-hydroxybenzoate production rate: 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 already improved *p*-hydroxybenzoate production by *P. putida* S12pal_xylB7 from glycerol was further enhanced by co-feeding of xylose. The product-to-substrate yield increased by a factor 1.9 to a maximum of 16.3 Cmol % (Fig. 2E). The q_p improved with Y_{ps} to a maximum value of 18 µmol C (g CDW)$^{-1}$ h^{-1}. Considering the relative improvements observed, the effect of xylose co-feeding was more pronounced in glycerol-grown cultures compared to glucose-grown cultures. This observation corroborates the beneficial effect of glycerol on *p*-hydroxybenzoate production by *P. putida* S12pal_xylB7. As was 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. 2F).

Effect of primary carbon source on xylose utilization

As discussed above, the mixed-feed chemostats were operated such that the primary carbon source was the limiting nutrient to ensure simultaneous utilization of xylose and the primary carbon source. However, the extent to which xylose was utilized was quite different for glucose and glycerol. Therefore, the relative xylose uptake was plotted as a function of the xylose in the feed (Fig. 3).

On glycerol-xylose mixtures, the amount of xylose in the feed correlated well with the amount of xylose actually consumed. Some residual xylose was observed, but the concentration was in the same range as observed in chemostat cultivations with xylose as the sole carbon source. This residual xylose concentration amounted to 0.27 mM, which is presumably close to the K_m of the transporter that is responsible for xylose import in *P. putida* S12pal_xylB7.

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. Thus, the capacity to transport and/or utilize xylose was apparently dependent on the type of primary carbon source, as well as on the relative amount of that primary carbon source in the feed. On glycerol, apparently no severe limitations for xylose utilization occurred, while on glucose such limitations did exist and were furthermore exacerbated with decreasing fractions of glucose in the feed.

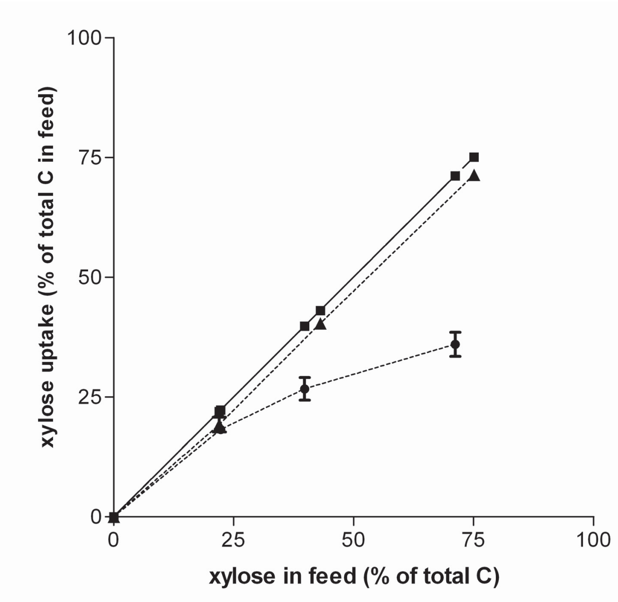


Figure 3. Relative xylose uptake as a function of the xylose fraction in the feed. The solid line represents the theoretical maximum uptake of xylose; the dotted lines represent the actual uptake of xylose with glycerol as co-substrate (triangles) or glucose as c-substrate (circles). Data are the average from two independent cultivations; error bars represent the maximum deviation from the mean.

Discussion

A novel strategy was devised to improve aromatics production by engineered *P. putida*. The approach was based on the assumption that the PP pathway intermediate E4P was a limiting factor for aromatic biosynthesis, and that the E4P availability could be improved by stimulating the PP pathway fluxes through pentose co-feeding. As *P. putida* is not capable of utilizing pentoses, a phosphorylative xylose pathway was introduced into a *p*-hydroxybenzoate producing *P. putida* S12 strain. The initially low growth rate on xylose was improved via an evolutionary selection procedure, which furthermore resulted in increased aromatic biosynthesis even in the absence of xylose. This improvement could presumably be attributed to an elevated PP pathway activity of the selected strain. Such a phenotype would have an evident selective advantage in the evolutionary engineering procedure and would additionally improve E4P availability.

The xylose co-feeding experiments confirmed the presumption that aromatics production would benefit from improved PP pathway fluxes. Considerable *p*-hydroxybenzoate yield improvements were observed, although the applied proportion of xylose in the feed apparently was of little effect within the range tested. This suggests either that E4P was no longer a limiting factor for aromatics production, or that the PP pathway was already fully saturated at the lowest relative xylose concentration applied.

Remarkably, both biomass and product yields were improved when glycerol was employed as the primary carbon source instead of glucose. The glycerol-associated product-yield improvement in *P. putida* S12pal_xylB7 may be associated with the anticipated improved PEP availability on glycerol. Moreover, it should be noted that the PEP-precursor G3P also feeds the PP pathway, both directly and via gluconeogenically produced fructose-6-phosphate (F6P) (Fig. 1). Thus, the favorable G3P stoichiometry on glycerol is likely to enhance E4P availability, through the elevated PP pathway activity in *P. putida* S12pal_xylB7. The G3P stoichiometry, however, does not explain the apparently improved overall carbon efficiency on glycerol. Recently, we have obtained indications that the glyoxylate shunt is active in glycerol-grown *P. putida* S12palB1 (Verhoef et al., manuscript in preparation) and thus, two CO_2 releasing steps in the citric acid cycle may be bypassed. In addition, glucose is preferably funnelled into the PP pathway via the oxidative catabolism of glucose-6-phosphate, rather than via F6P and G3P (1, 4). This oxidative pentose shunt results in the formation of an additional CO_2. Thus, the primary metabolism on glycerol appears to generate less CO_2, resulting in an increased biomass and product yield.

The different G3P stoichiometries for glucose and glycerol may also provide an explanation for the effect of the primary carbon source on xylose uptake. As discussed, G3P is the precursor of PEP which is involved in bacterial sugar transport via the phosphotransferase system (PTS; for reviews, see (3, 13)). Fig. 3 shows a primary-carbon-source dependency of xylose uptake, inferring that xylose import is PEP-dependent. Since fructose is the only identified PTS sugar in *P. putida* (6), it may be assumed that xylose uptake is facilitated by the fructose transporter. It should be noted that *P. putida* S12pal_xylB7 was derived from strain S12_427, which was obtained after random mutagenesis and antimetabolite screening with fructose as the carbon source (23). Thus, a mutant fructose transporter may have been selected for with an –unintentionally- improved affinity for xylose. The role of the heterologous ATP-dependent xylose transporter XylFGH was presumably limited, as has been remarked previously (9). Since the total amount of energy derived from glucose and glycerol is similar, ATP-dependent xylose-import is unlikely to account for the observed xylose uptake behavior. Moreover, PEP-dependent xylose-import would agree with the dramatically decreased growth rate on xylose upon introducing Pal/Tal. The resulting aromatics biosynthesis then causes a drain on PEP that would obviously interfere with xylose import and growth on xylose.

It was clearly demonstrated that the approach of co-feeding different carbon sources to stimulate the fluxes of different pathways is a powerful tool to engineer microbial production of aromatics. This concept may be more widely applied to improve the production efficiency of other compounds that are derived from other primary pathways. Different co-substrates may be employed or the point of entry of a specific substrate in the primary metabolism may be engineered. We recently demonstrated this principle by directing xylose towards the citric acid cycle instead of the PP pathway, by employing an oxidative catabolic pathway from *Caulobacter crescentus* (10). For further improvement of aromatics production, engineering the G3P / PEP availability appears to be a promising target. An ED-negative glycolytic *P. putida* S12 strain may be constructed and the presumed PEP-dependent xylose transporter may be replaced with a PEP-independent transporter. Furthermore, the overall carbon efficiency, or more specifically the product-to-substrate yield, may be improved by eliminating or downtuning the oxidative pentose shunt, bypassing the citric acid cycle via the glyoxylate shunt and stimulating anaplerosis via pyruvate carboxylase.

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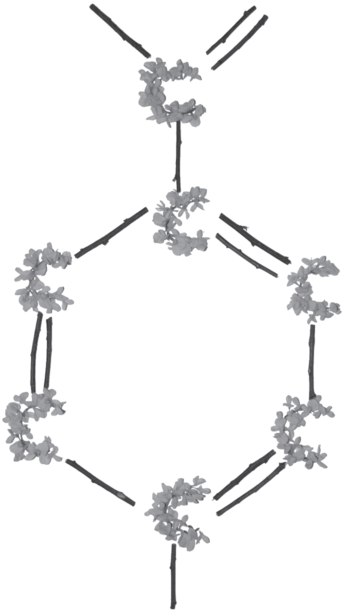
Chapter 5

Crude glycerol as feedstock for the production of substituted aromatics: a case for *Pseudomonas putida* S12



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Verhoef S, Gao N, de Winde JH and Ruijssenaars HJ Crude glycerol as feedstock for the production of substituted aromatics: a case for *Pseudomonas putida* S12.

Abstract

The use of crude, unpurified glycerol as substrate for *Pseudomonas putida* S12 and *Escherichia coli* DH5α was evaluated. In high-cell density fed-batch fermentations, *P. putida* S12 performed consistently better on crude glycerol than on purified glycerol, as shown by the biomass to substrate yield, maximum biomass production rate and substrate uptake rate. Also the production of *p*-hydroxybenzoate by an engineered *P. putida*, strain S12palB5, was more efficient on crude glycerol, with a yield of 6.6 % (C-mol product / C-mol glycerol) compared to 5.9 % on purified glycerol. By contrast, *E. coli* DH5α showed decreased biomass yield and growth rate on crude glycerol compared to purified glycerol, and increased acetate formation (11.5 and 16.2 g l^{-1} on purified, respectively, crude glycerol). It was concluded that *P. putida* S12 is a more suitable host than *E. coli* for crude glycerol-based bioproduction processes.

Introduction

In recent years, biodiesel has rapidly become an increasingly attractive renewable fuel. Inevitably, for each gallon of biodiesel produced, approximately 0.3 kg of glycerol (around 10 % by weight) is generated (26). Due to the rapid growth of the biodiesel industry, crude glycerol is increasingly becoming a cheap and abundant carbon source (11) fuelling interest in opportunities to convert glycerol into higher-value products (3, 11, 18, 34). A promising outlet is the use of crude glycerol as a renewable feedstock in bioconversion processes for the production of fuels and (base) chemicals (10, 19, 20, 23, 25, 35). However, the presence of considerable amounts of impurities in crude glycerol such as methanol, free fatty acids, and salts (especially sodium or potassium) may strongly interfere with the fermentation process (10, 20, 23). Hence, the utility of this renewable fermentation feedstock is limited unless a robust fermentation host is employed.

In this study, we evaluated the use of crude glycerol as potential carbon source for the bioproduction of substituted aromatics. These compounds can be produced by engineered solvent-tolerant *Pseudomonas putida* strains (22, 28, 29, 33) or engineered *Escherichia coli* strains (1, 21). The growth performance of both *P. putida* S12 and *E. coli* DH5α were compared on crude and purified glycerol and, as a demonstrator case, the production of *p*-hydroxybenzoate by *P. putida* S12palB5 from crude glycerol was investigated.

Materials and methods

Bacterial strains and culture conditions

The following strains were used in this study: wild type *P. putida* S12 (ATCC 700801), a *p*-hydroxybenzoate producing strain *P. putida* S12palB5 (*P. putida* S12palB1 (28) containing an inactivated *hpd* gene (27) and *Escherichia coli* DH5α (Invitrogen). Shake flask experiments were performed in 250-ml Erlenmeyer flasks containing 50 ml of a phosphate buffered mineral salts medium adapted from a previously described medium (28) containing 3.88 g l^{-1} K_2HPO_4 and 1.63 g l^{-1} NaH_2PO_4, supplemented with 40 mM of either purified glycerol (MMGly) or crude glycerol (MMRGly). For *E. coli*, thiamine (25 mg l^{-1}) was added to the minimal medium. To cultures of *P. putida* S12palB5, 0.1 mM of sodium salicylate and 10 µg ml^{-1} of gentamicin were added. Cultures were inoculated to a starting optical density at 600 nm (OD_{600}) of 0.2 with cells from a late-log phase preculture on MMGly. The *P. putida* S12 strains were grown at 30 °C, while *E. coli* DH5α was grown at 37 °C.

Fed-batch experiments were performed in 2-l fermentors (New Brunswick Scientific) using a BioFlo110 controller. The initial batch phase was started with 50 ml of a preculture on MMGly. For the batch phase, 1 l of mineral salts medium was used with the following composition (per l): (crude) glycerol, 18.4 g; $(NH_4)_2SO_4$, 2 g; K_2HPO_4, 3.88 g; $NaH_2PO_4 \cdot H_2O$, 1.63 g and 20 ml of trace element solution (28). After depletion of the initially supplied glycerol, the feed was started and controlled to allow maximum growth while maintaining glycerol as the limiting substrate in the culture. The feed solution contained (per l): (crude) glycerol, 736 g and $MgCl_2{\cdot}6H_2O$, 10 g. For *P. putida* S12palB5, gentamicin (10 mg l^{-1}) and sodium salicylate (0.1 mM) were added to the batch culture and the feed solution. For *E. coli*, 25 mg of thiamine was added during the batch phase and after 30 h of cultivation another 25 mg of thiamine was added to the culture broth.

The initial stirring speed was set to 200 rpm and air was supplied to the head space at 1 l min^{-1} using a M+W Instruments D-5111 mass-flow controller. Dissolved oxygen tension (DO) was continuously monitored with an InPro model 6900 probe and maintained at 30 % air saturation by automatic adjustment of the stirring speed to a maximum of 1000 rpm. When the maximum stirring speed was reached, air was replaced with purified oxygen at a flow of 0.2 l min^{-1} and the maximum stirring speed was set to 800 rpm. The pH was maintained at 7.0 by automatic addition of 25 % NH_4OH and the temperature was kept at 30 °C for *P. putida* S12 strains and 37 °C for *E. coli* DH5α. Samples were drawn during the culture to determine cell density and concentrations of glycerol, acetate, *p*-hydroxybenzoate and ammonium.

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). Glycerol and acetate were analyzed by ion chromatography (Dionex ICS3000 system), using an IonPac ICE AS1 column with 100 mM methyl sulphonic acid as eluent for glycerol or an IonPac ICE AS6 column with 0.4 mM heptafluorobutyric acid as the eluent for acetate. The *p*-hydroxybenzoate concentration was measured as described previously (28). Elemental analysis of crude glycerol was performed by inductively coupled plasma optical emission spectroscopy (ICP-OES; Optima 5300dv, PerkinElmer Inc.). Prior to analysis, crude glycerol was diluted 100 times in 0.5 M nitric acid. The calibration samples for the different elements were supplemented with purified glycerol to a final concentration of 10 g l^{-1} to exclude artifacts due to the presence of glycerol in the crude glycerol sample. NH_4^+ concentrations were determined as described previously (29).

Chemicals

Crude glycerol (~80 % glycerol content; sample number A-5106) was kindly provided by Cremer Oleo GmbH & Co. KG, Hamburg, Germany. Analytical grade (purified) glycerol was purchased from Fisher Emergo BV, The Netherlands. Glycerol, both crude and purified, was sterilized by autoclaving for 18 min at 105 °C. Stock solutions for sodium salicylate and thiamine were prepared and filter sterilized just before use. All other medium components were autoclaved for 20 min at 121 °C.

Results

Crude glycerol: composition and evaluation as substrate in shake flask cultures

Crude glycerol contains various impurities like methanol, salts and fatty acids (26). In the present study, rapeseed-based crude glycerol was used that contained 80 % glycerol, max. 10 % ash, max. 3 % non-glycerol organic matter, max. 0.5 % methanol and water, according to the product specifications. This composition was in line with the values published by Thompson and He (26). The actual glycerol concentration was established to be 1.0 kg l^{-1} which corresponds to a glycerol content of 79 % (w/v). The ash content was analyzed and

Table 1. Metal concentrations in crude glycerol and liquid glycerol media in mg l^{-1}.

	Na	K	Ca	Mg	Fe	Mo	Zn	Cu	Mn	Co
Crude glycerol (80%)[a]	39100	512	48.9	45.1	3.5	2.3	1.5	<1[d]	<1[d]	ND[e]
MMGly[b]	314	1738	0.27	12	1	0.08	0.45	0.05	0.34	0.1
MMRGly[c]	443	1740	0.43	12.1	1.01	0.09	0.46	0.05[f]	0.34[f]	0.1[f]

[a] measured with elemental analysis

[b] calculated based on the mineral salts medium composition

[c] calculated based on the mineral salts medium composition and on the crude glycerol composition for 40 mM of crude glycerol

[d] below detection limit

[e] ND: not determined

[f] assuming that this element is not present in crude glycerol

MMGly: mineral salts medium with 40 mM glycerol; MMRGly: mineral salts medium with 40 mM crude glycerol

found to consist mainly of sodium salts (sodium ion concentration 39 g l^{-1}, i.e., 3.1 % (w/v)), while the other elements analyzed were present only in trace amounts (Table 1).
The ability of *P. putida* S12 and *E. coli* DH5α to use crude glycerol as a substrate was tested in shake flask cultivations. Both *P. putida* S12 and *E. coli* DH5α were able to grow in mineral salts medium supplemented with 40 mM of crude glycerol. For either strain, no pronounced differences in growth were observed between the crude glycerol cultures and control cultures on purified glycerol. The elemental composition of the glycerol media was not much affected by the addition of 40 mM crude glycerol as shown in Table 1. Nevertheless, crude glycerol could replace the trace element solution, with the exception of magnesium and iron, to sustain growth of *P. putida* S12.

Fed-batch cultivations on purified and crude glycerol

Crude glycerol did not have an adverse effect on bacterial growth in shake flask cultures, which can be attributed to the low level of potential growth inhibitors present at the crude glycerol concentrations employed. In industrial fermentations, however, concentrated feed streams are applied in fed-batch cultivations, which will result in the accumulation of feedstock-associated fermentation inhibitors. Therefore, fed-batch cultivations with crude and purified glycerol feeds were performed for both wild type *P. putida* S12 (Fig. 1A) and *E. coli* DH5α (Fig. 1B). In addition, *P. putida* S12palB5 was cultivated in fed-batch mode (Fig. 1C) to study the effect of crude or purified glycerol on *p*-hydroxybenzoate production.
The fed-batch cultivations of both wild type *P. putida* S12 and *P. putida* S12palB5 exhibited similar behaviour on crude glycerol compared to purified glycerol: both reached a higher final biomass density, maximum growth rate and substrate uptake rate (Table 2). The biomass yield Y_{xs} appeared higher for crude glycerol, when calculated for the amount of glycerol consumed. However, crude glycerol also contains other organic matter, such as fatty acids (26). When also the non-glycerol carbon was taken into account, the yields on crude and purified glycerol were comparable for the wild-type strain, and slightly elevated on crude glycerol for the *p*-hydroxybenzoate producing strain. It should be noted that carbon limitation was not maintained during the feeding phase in the cultures of wild type *P. putida* S12 (Fig. 1A). The potential effects of the excess glycerol were considered negligible, however, since *P. putida* S12 shows no overflow metabolism in the presence of excess carbon and the specific

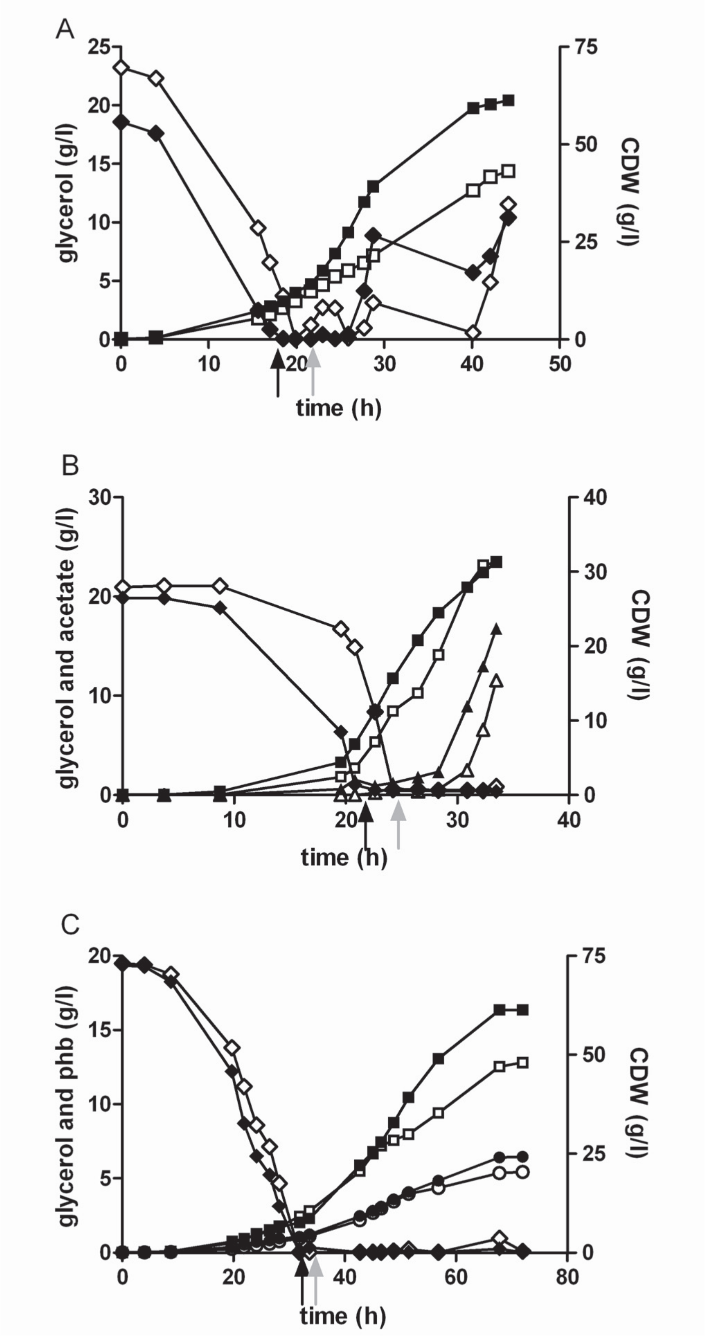

Figure 1. Carbon limited fed-batch cultivations on purified (open symbols) and crude glycerol (closed symbols) of P. putida *S12 (A),* E. coli *DH5α (B) and* P. putida *S12palB5 (C). Cell dry weight (CDW) (□/■), concentration glycerol (◊/♦), concentration acetate* (Δ/▲) *and concentration p-hydroxybenzoate (○●). The arrows indicate the start of the glycerol feed (filled: crude glycerol, grey: purified glycerol). The data presented are from a single representative experiment.*

substrate uptake rate was unaltered.

In contrast to the consistently improved performance of both *P. putida* S12 strains on crude glycerol, the performance of *E. coli* DH5α was consistently compromised. Biomass yield (calculated either on a glycerol or on a total carbon basis) and growth rate were considerably lower on crude glycerol. Also the final biomass density was lower, although the substrate uptake rate was higher for crude glycerol. The overall poor performance of *E. coli* DH5α compared to *P. putida* with glycerol as the substrate may be attributed mostly to acetate formation, which lowered the biomass yield as less substrate was available for biomass formation. Also the observed growth inhibition may be attributed to the toxic effects exerted by acetate accumulation (4, 15). The accumulation of acetate was more pronounced on crude glycerol. This is illustrated by the acetate yield on glycerol, being 0.12 and 0.15 g g^{-1} on purified and crude glycerol. Combined with the higher substrate uptake rate, this phenomenon led to a considerably higher final acetate concentration on crude glycerol: 16.2 g l^{-1} compared to 11.5 g l^{-1} on purified glycerol.

The growth performance of *P. putida* S12 was positively affected by crude glycerol, and also *p*-hydroxybenzoate production by *P. putida* S12palB5 was improved. The final product concentration (Fig. 1C), as well as the product to substrate yield (Y_{ps}), was higher on crude glycerol compared to purified glycerol (Table 2). The product to biomass yield (Y_{px}) was slightly higher on purified glycerol, however, which can be attributed to the elevated biomass yield on crude glycerol, also when corrected for the consumption of non-glycerol carbon. On

Table 2. Characteristics of fed-batch cultivations on purified and crude glycerol as substrate of P. putida *S12,* P. putida *S12palB5 and* E. coli *DH5α.*

	P. putida S12		*P. putida* S12palB5		*E. coli* DH5α	
	purified	crude	purified	crude	purified	crude
CDW (g l^{-1})	43.7±0.9	59.9±2.1	47.3±1.0	55.6±7.9	32.8±2.1	30.9±0.7
Y_{xs} (Cmol%)[a]	54.8±0.5	58.8±0.6	43.2±0.8	50.7±0.3	45.8±0.9	42±0.6
Y_{xs*} (Cmol%)[b]		54.9±0.6		47.4±0.3		39.2±0.6
Y_{ps} (Cmol%)[a]			5.9±0.2	6.6±0.1		
Y_{ps*} (Cmol%)[b]				6.1±0.1		
Y_{px} (Cmol%)			13.6±0.2	12.9±0.2		
$r_{x,max}$ (Cmol h^{-1})	0.10±0.01	0.17±0.02	0.087±0.01	0.10±0.02	0.18±0.03	0.12±0.008
$r_{s,max}$ (Cmol h^{-1})[a]	0.19±0.01	0.26±0.02	0.16±0.012	0.19±0.002	0.34±0.02	0.43±0.04
$r_{p,max}$ (Cmol h^{-1})			0.012±0.0002	0.012±0.001		
p-hydroxybenzoate (g l^{-1})			5.3±0.2	6.0±0.6		
Acetate (g l^{-1})					11.5±1.2	16.2±0.8
Na^+ (mM)[c]	12.4±0.4	285±5	11.4±0.2	246±63	11.8±0.7	170±4

Data are from two representative fed-batch cultivations, errors represent the maximum deviation from the mean.

[a] yield calculated from the consumed amount of glycerol

[b] corrected yield, calculated based on total amount of carbon present; assumed that the 3 % organic matter (non-glycerol) has all been utilized and consists only of fatty acids with an average chain length of 20 (26) which resulted in 7 % more available carbon. Furthermore, no carbon contribution was expected from the 0.5 % methanol present, since it has assumed to be evaporated during the autoclaving process

[c] calculated based on the amount of crude glycerol added and the elemental composition of crude glycerol (see Table 1)

the other hand, the maximum *p*-hydroxybenzoate production rate ($r_{p,max}$) was identical for both carbon sources (Table 2). The lower Y_{xs} of the production strain *P. putida* S12palB5 compared to wild type *P. putida* S12 could largely be ascribed to the production of *p*-hydroxybenzoate, which draws on biosynthetic intermediates but also induces a solvent stress-response that results in an additional energy demand (8, 27, 31). The final sodium concentrations for the crude glycerol fermentations were estimated from the sodium content of the crude glycerol stock (Table 1) and the amount of crude glycerol added to the fermentation. Table 2 shows that the final sodium concentrations were 14 to 23-fold higher in the crude glycerol fed-batches fermentations. For purified glycerol as substrate, only the added buffer and medium salts contributed to the final amount of sodium.

Discussion

In the present study, marked differences were observed in the performance of *P. putida* S12 and *E. coli* DH5α on crude glycerol as substrate. At low crude glycerol concentrations, no negative effects on growth were observed for *P. putida* S12. Also *E. coli* was not inhibited by low crude glycerol concentrations, which is in agreement with a recent report by Yazdani and Gonzalez (35). The inhibiting effect of crude glycerol on *E. coli* became truly apparent during fed-batch cultivation, in which inhibiting compounds could accumulate. In contrast, *P. putida* S12 consistently performed better on crude glycerol compared to purified glycerol. The improved performance was observed with respect to both growth and *p*-hydroxybenzoate production, confirming previous observations that growth and aromatics biosynthesis are tightly coupled in engineered *P. putida* S12 (28, 29, 33).

The improved performance of *P. putida* S12 on crude glycerol suggests that salts or other inhibitors present in crude glycerol did not cause stress in this bacterium under the conditions investigated. This observation is in agreement with the reported tolerance of *P. putida* S12 to diverse types of aggressive compounds, ranging from organic solvents and antibiotics to heavy metals (5, 7, 9, 13, 30, 32). This intrinsic robustness expectedly plays a role in the tolerance to crude glycerol, but does not explain why the performance was actually improved. Since *P. putida* is capable of utilizing long chain fatty acids (6, 24), growth was possibly stimulated by the fatty acids that are commonly present as impurity in crude glycerol (26).

The relatively poor performance of *E. coli* DH5α in fed batch cultivations on glycerol can be attributed mostly to extensive acetate accumulation. Acetate formation by aerobically-grown *E. coli* is a well-known phenomenon that relates to the inability of the respiratory system to keep up with carbon metabolism, which results in the fermentation of excess carbon to acetate (4). Indeed, additional feeding of glycerol in growth-arrested fed-batch cultures resulted in further accumulation of acetate rather than resumption of cell growth (unpublished data). In addition to lowering the biomass yield, acetate accumulation inhibits growth. In glucose-grown batch cultivations the specific growth rate has been reported to decrease by 70 % at 15 g l^{-1} acetate (16). The formation of acetate was exacerbated when crude glycerol was used as the substrate. This phenomenon is partly accounted for by the higher substrate uptake rate on crude glycerol that, paradoxically, suggests that crude glycerol is actually a better substrate for *E. coli*, possibly for the same –hypothetical- reasons as for *P. putida*. Since the respiratory capacity is the limiting factor in *E. coli*, however, the increased substrate uptake rate could only result in additional acetate accumulation instead of improved growth.

Furthermore, the presence of inhibitors may cause additional stress contributing to increased acetate formation. In principle, acetate formation can be overcome by lowering the specific growth rate to below a critical value and avoiding oxygen-limited conditions (4, 14). High cell density cultivations of *E. coli* on glycerol are possible up to 180 g l^{-1} CDW (16), but the cultivation time is increased due to the lower growth rate that must be imposed.

The accumulation of salt in fed-batch cultures on crude glycerol was expected to have negative effects, since high sodium concentrations have been reported to affect the growth rate of both *E. coli* (2) and *P. putida* S12 (12). The observation that growth of *E. coli* DH5α ceased at considerably lower salt concentrations than *P. putida* S12, suggests that *E. coli* suffers more from the accumulation of salt or other impurities, or from the combined effects of impurities and acetate accumulation. Although a high salt concentration may have a negative effect on growth rate, it may be beneficial for product recovery since it facilitates salting-out extraction (17).

Crude glycerol was shown to be a suitable substrate for growth and aromatics production by *P. putida* S12 whereas for *E. coli*, crude glycerol is an inferior substrate. This may –in part- be alleviated through tight control of fermentation conditions, at the cost of a significantly increased process time and, therefore, decreased productivity. The tolerance of *P. putida* S12 towards chemical aggression, its high respiratory capacity, and the absence of overflow metabolites makes this strain intrinsically more suitable as bioproduction host for crude glycerol-based bioconversion processes.

Acknowledgements

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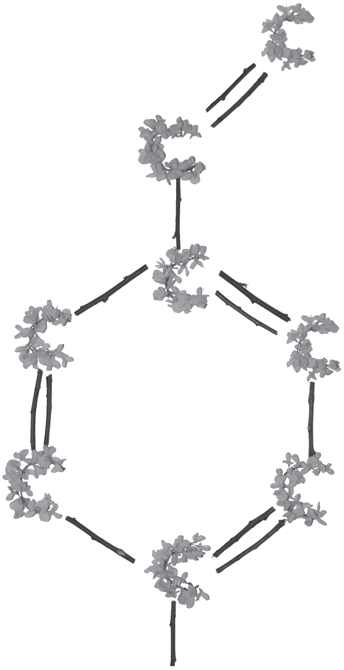
Chapter 6

Bioproduction of *p*-hydroxystyrene from glucose by solvent-tolerant *Pseudomonas putida* S12 in a two-phase water-decanol fermentation



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[*] Both authors contributed equally to this work

Abstract

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/tal* and *pdc*, respectively encoding phenylalanine/tyrosine ammonia lyase and *p*-coumaric acid decarboxylase. These enzymes allow the conversion of the central metabolite tyrosine into *p*-hydroxystyrene, via *p*-coumarate. Degradation of the *p*-coumarate intermediate was prevented by inactivating the *fcs* gene encoding feruloyl-CoA synthetase. The best performing strain was selected and cultivated in fed-batch mode resulting in the formation of 4.5 mM of *p*-hydroxystyrene at a yield of 6.7% (C-mol *p*-hydroxystyrene per C-mol 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.

Introduction

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 emission (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 which 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 towards 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 (encoded by *pal/tal*) and *p*-coumaric acid decarboxylase (encoded by *pdc*) from *Rhodotorula glutinis* and *Lactobacillus plantarum*, respectively, were introduced for the conversion of tyrosine into *p*-hydroxystyrene via *p*-coumarate. The maximum concentration of *p*-hydroxystyrene was limited to 3.3 mM due to

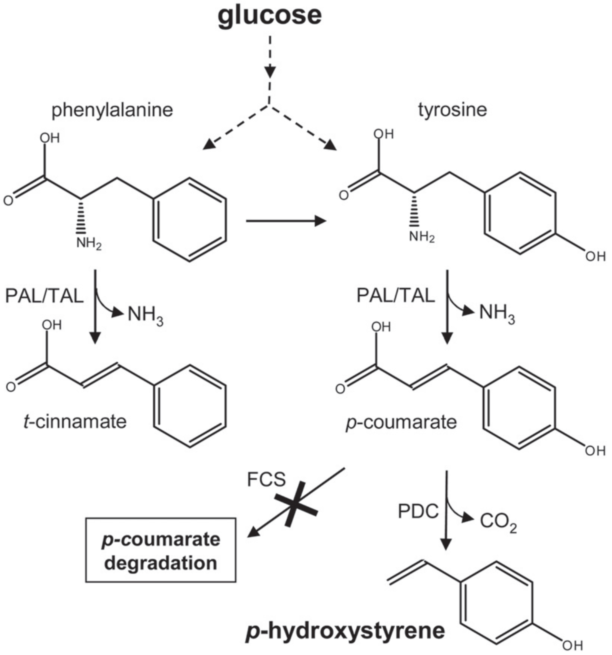

Figure 1. Schematic overview of the biochemical pathway for p-*hydroxystyrene production. Pal/Tal, phenylalanine/tyrosine ammonia lyase; PDC,* p-*coumaric acid decarboxylase; Fcs, feruloyl-CoA synthetase. The cross indicates the disruption of* fcs*, disabling* p-*coumarate degradation.*

the toxicity of the product to the *E. coli* host (3, 23). To alleviate product toxicity, a two-phase fermentation with 2-undecanone as 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 tyrosine from glucose by *E. coli*, conversion of 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 tyrosine derived aromatics *p*-coumarate and phenol, respectively. These strains were highly optimized for aromatics production, resulting in a heavily increased metabolic flux towards tyrosine. Therefore, they are suitable platform strains for the production of other tyrosine derived aromatics (33). The bifunctional 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 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 mineral salts media 20 mM of glucose (MMG) was used as the sole carbon source. For cultivation of phenylalanine auxotrophic strains, 10 mg liter^{-1} phenylalanine was added to the medium. The expression of the introduced copy of *aroF-1* in derivatives of strain 427 as well as the expression of *palpdc* in the pJNTpalpdc vector were induced by addition of 0.1 mM of sodium salicylate. Antibiotics were added as required to the media 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}; 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 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 MMG with gentamicin and salicylate. The batch phase for both the single and two-phase fermentations was started with

Table 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics [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 427Δfcs	*fcs* knockout strain of *P. putida* S12 427	This study
P. putida S12 427Δfcs pJNTpalpdc	*fcs* knockout strain of *P. putida* S12 427 containing plasmid pJNTpalpdc	This study
P. putida S12 427Δfcs 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 C3Δsmo	*smo* knockout strain of *P. putida* S12 C3	This study
P. putida S12 C3Δsmo pJNTpalpdc	*smo* knockout strain of *P. putida* S12 C3 containing plasmid pJNTpalpdc	This study
P. putida S12 C3Δsmo pJT'Tpalpdc	*smo* knockout strain of *P. putida* S12 C3 containing plasmid pJT'Tpalpdc	This study
Escherichia coli DH5α	*supE*44 Δ*lac*U169 (φ80 *lac*ZΔM15) *hsdR*17 *rec*A1 *endA*1 *gyrA*96 *thi*-1 *relA*1	(26)
Lactobacillus plantarum	Source of *pdc,* DSMZ20174	(31)
Plasmids		
pTacpal	Ap^r Gm^r, expression vector containing the *pal* gene under 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 control of the *tac* promoter and *tac* RBS	Unpublished data
pJNTpal	Ap^r Gm^r, pJNT containing the *pal* gene under 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 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 control of the *tac* promoter and *tac* RBS	This study
pJQ200SK	Suicide vector, P15A *ori sac*B 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] Ap^r, Gm^r, Km^r and Tc^r, ampicillin, gentamicin, kanamycin and tetracycline resistance respectively.

[b] previous known as *P. putida* S12 TPL3c.

1.5 liter medium of the following composition: glucose 13.5 g, $(NH_4)_2SO_4$ 1.5 g, K_2HPO_4 5.82 g, $NaH_2PO_4.H_2O$ 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 (liter^{-1}): glucose 158 g, $(NH_4)_2SO_4$ 16.5 g, K_2HPO_4 3.88 g, $NaH_2PO_4.H_2O$ 1.63 g, gentamicin 10 mg, sodium salicylate 1 mmol, $MgCl_2$ 1.5 g and 0.1 liter trace element solution. The feed for the two-phase fermentation contained (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 liter trace element solution. Samples were drawn during the culture to determine 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 $liter^{-1}$ 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 HPLC (Agilent 1100 system) using a Zorbax SB-C18 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 the total *p*-hydroxystyrene concentration, respectively, the concentrations in the aqueous and the 1-decanol phase. V_{tot}, V_{aq} and V_{dec} are the total liquid volume, respectively the volumes of the aqueous and the 1-decanol phase.

DNA techniques

Plasmids were introduced into *P. putida* S12 by electroporation using a Gene Pulser electroporation device (Biorad). The targeted gene disruption of the *fcs* gene in *P. putida* S12 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 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

Table 2. Oligonucliotide primers used in this study

No	Sequence (5’ → 3’)[a]	Characteristics
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 *Lactobacillus plantarum*, forward primer
8	gcggctagcttacttatttaaacgatggtagttttg	End of *pdc* from *Lactobacillis plantarum*, reverse primer

[a] Restriction sites are underlined.

LB-agar plates containing 1 mM of indole, and induced by air saturated with styrene for SMO induction. Native SMO converts indole into indigo resulting in blue coloured 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 *Not*I and *Nhe*I 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 styrene monooxygenase (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 C3Δsmo. Wild type *P. putida* S12 degraded *p*-hydroxystyrene, but only in the presence of styrene. *P. putida* S12 C3Δsmo did not degrade *p*-hydroxystyrene, either in 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 towards tyrosine, *P. putida* S12 C3 and *P. putida* S12 427, were modified to produce *p*-hydroxystyrene via 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-CoA

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

Strain	Cultivation	Max [pHS] (mM)	$Y_{p/s}$ (C-mol %)	$r_{p,max}$ (mM.h^{-1})
S12 C3Δsmo pJT'Tpalpdc	Shake flask	0.35	2.3	0.01
S12 C3Δsmo pJNTpalpdc	Shake flask	0.72	4.8	0.03
S12 427Δfcs pJT'Tpalpdc	Shake flask	0.88	5.9	0.1
S12 427Δfcs pJNTpalpdc	Shake flask	1.1	7.4	0.1
S12 427Δfcs pJNTpalpdc	Single-phase fed batch	4.5	6.7	0.4
S12 427Δfcs pJNTpalpdc	Biphasic fed batch	21[a]	4.1	0.75

Max [pHS] maximum concentration *p*-hydroxystyrene, $Y_{p/s}$ product to substrate yield in C-mol *p*-hydroxystyrene per C-mol glucose, $r_{p,max}$ maximum volumetric production rate.

[a] For the calculation see section material and methods.

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 C3Δsmo and *P. putida* S12 427Δfcs by transformation of one of the *pal-pdc* expression plasmids pJNTpalpdc or pJT'Tpalpdc, enabling the conversion of 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 in shake flask cultures (Table 3).

Both *P. putida* S12 427 derived strains show a higher product to substrate yield (Y_{ps}) 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 427Δfcs 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 427Δfcs pJNTpalpdc was studied in fed-batch cultures (Fig. 2). *p*-Hydroxystyrene accumulated to a maximum concentration of 4.5 mM with a Y_{ps} of 6.7 C-mol%, a biomass to substrate yield (Y_{xs}) of 50 C-mol% and a product to biomass yield (Y_{px}) 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

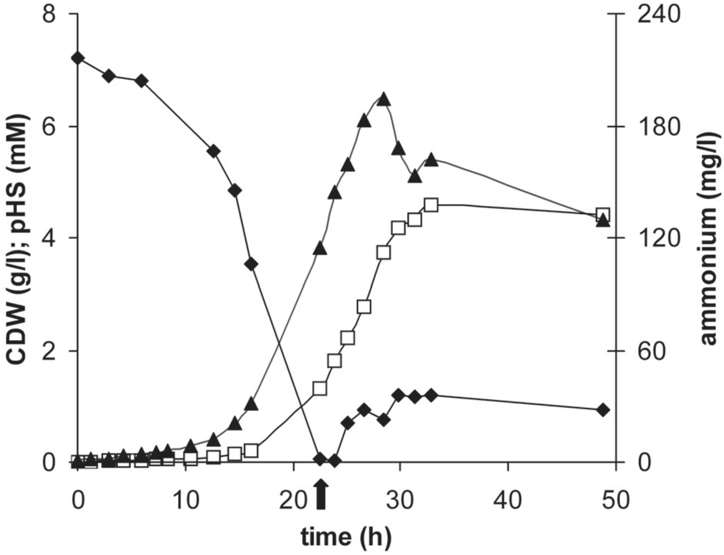

Figure 2. Production of p-*hydroxystyrene by* P. putida *S12 427Δfcs pJNTpalpdc in fed-batch cultivation. Concentration* p-*hydroxystyrene (pHS, □), cell dry weight (CDW) (▲) and ammonium (♦). The arrow indicates the time that the feed was started. The data presented are from a single representative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

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

Upon reaching a concentration of 4.5 mM *p*-hydroxystyrene, growth and *p*-hydroxystyrene production were completely halted and lysis occurred (Fig. 2). Two-fold 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 427Δfcs 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 1-decanol after 15 hours of cultivation (Fig. 3). Also, the feed was concentrated two-fold in order to compensate for the volume that the 1-decanol phase occupies in the bioreactor.

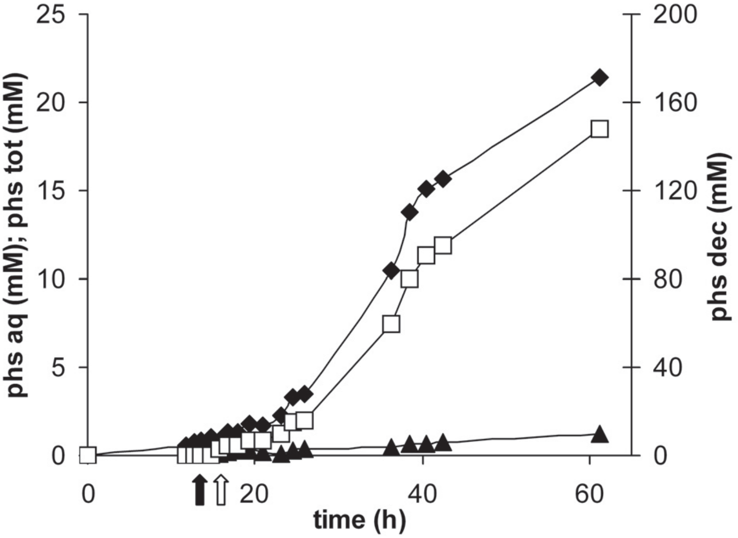

Figure 3. Production of p-*hydroxystyrene by* P. putida *S12 427Δfcs pJNTpalpdc during a two-phase water-1-decanol nitrogen limited fed-batch fermentation. Total concentration* p-*hydroxystyrene (phs tot) (♦, left Y-axis) calculated as described in material and method section, concentration* p-*hydroxystyrene in the 1-decanol phase (phs dec) (□, right Y-axis) and concentration* p-*hydroxystyrene in the aqueous phase (phs aq) (▲, left Y-axis). 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.*

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 where 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 phase. 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 (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 or 2- ketogluconate were 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 presenceof 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_{px} is 13 C-mol%. The Y_{xs} and Y_{ps} 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, co-utilization of 1-decanol in the biphasic culture can not 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 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 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 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_{ps} and $r_{p,max}$ compared to strain C3 derivatives (Table 3). The latter three factors were regarded as more important for efficient production and thus *P. putida* S12427Δfcs 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_{ps} for *p*-hydroxystyrene was approximately 1.5-fold lower than for the less toxic product *p*-coumarate ((19), 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 427Δfcs 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 already 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_{ps}. The negative impact of solvents on Y_{xs} 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-type solvent extrusion pumps (13, 17). Since biomass and product formation are closely linked, a decreased Y_{xs} will translate directly into a lower Y_{ps} 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_{xs} caused by the extractant toxicity may be averted by the physical separation of the aqueous and organic phases with 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 solvent to water ratio, reactor volume and 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 economical benefit.

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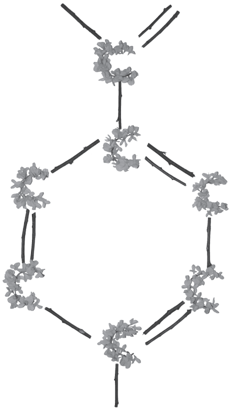
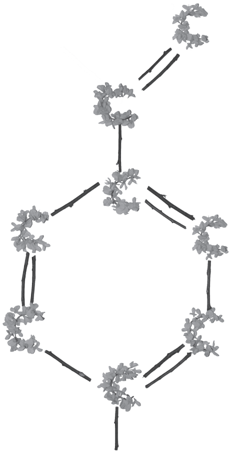
Chapter 7

General discussion and outlook





General discussion and outlook

The work described in this thesis exemplified the biological production of the hydroxylated aromatic compounds *p*-hydroxybenzoate and *p*-hydroxystyrene by engineered *P. putida* S12 strains. The different Chapters describe various approaches, from redirecting the carbon fluxes by introducing and deleting genes to co-feeding strategies and two-phase water-organic solvent cultivation, which resulted in improved production characteristics. Furthermore, system-wide analysis techniques at the transcriptome and proteome level were successfully applied to unravel the molecular and physiological background of the improved production. In addition, these methods have yielded valuable insights, of which some were pursued for further rational strain improvement. In this Chapter the effects of the different optimization strategies will be discussed in view of a challenging question: is *P. putida* S12 actually the preferred host for aromatics production from renewable feedstock?

Various strategies to improve aromatics production in *P. putida* S12

The production of hydroxylated aromatics by *P. putida* S12 was improved by applying both knowledge-driven approaches and experiment-driven leads. Although the applied optimization approaches were mainly tested for *p*-hydroxybenzoate production, most of these strategies are also likely to be appropriate to improve the biosynthesis of tyrosine derived aromatics. Table 1 presents an overview of the various optimization steps and the resulting product-to-substrate yields (Y_{ps}) and product titers.

The availability of the genome sequence of the related strain *P. putida* KT2440 (11, 32) has simplified several steps of the optimization strategy including biosynthetic pathway design and targeted disruption of genes (Chapter 2, 3 and 6). As demonstrated in this thesis, the *p*-hydroxybenzoate biosynthetic pathway designed and based on the genome sequence of *P. putida* KT2440 was appropriate for *P. putida* S12. Nevertheless, although *p*-hydroxybenzoate synthesis was enabled through (over) expression of the heterologous enzyme Pal/Tal (phenylalanine/tyrosine ammonia lyase) while *p*-hydroxybenzoate degradation was prevented, hardly any *p*-hydroxybenzoate was initially produced from glucose (Table 1). The metabolic network of microorganisms is usually not optimized for overproducing a single compound (5, 23) and apparently the carbon flux towards *p*-hydroxybenzoate was relatively low. Previous research on biosynthesis of aromatic compounds has resulted in the molecular engineering of *P. putida* S12_427, which has an optimized carbon flux towards tyrosine (33). By employing this strain for *p*-hydroxybenzoate production, the product-to-substrate yield increased by roughly a factor 100 (Table 1). Thus, besides the construction of the biosynthetic pathway, the metabolic flux towards the product is of crucial importance. Based on insights obtained from the transcriptomics analysis (Chapter 3) and a metabolic flux analysis (unpublished data) of the *p*-hydroxybenzoate producing strain, the *p*-hydroxybenzoate yield was further improved to a maximum value of 17.5 Cmol % on glucose (Table 1) and 19.3 Cmol % on glycerol (Chapter 4).

Although the product-to-substrate yield was substantially improved in this thesis, still further strain improvement is possible. In Chapter 4 it was suggested that the flux through the tyrosine biosynthesis pathway is a possible bottleneck for *p*-hydroxybenzoate production. As

Table 1: Optimization strategies for p-*hydroxybenzoate production by engineered* P. putida *S12 and the resulting product-to-substrate yields (Y_{ps}) and product titers.*

Goal	Approach	Y_{ps} (Cmol %)[a]	Product titer (mM)[a]	Reference
Establishing the biosynthetic pathway from a renewable substrate to *p*-hydroxybenzoate	Introduction of the gene *pal/tal*	0.0	0.0	Chapter 2
Preventing *p*-hydroxybenzoate degradation	Targeted interruption of the gene *pobA*	0.1	0.02	Unpublished data
Enhancing the carbon flux to tyrosine	Overexpressing of the gene *aroF-1*, random mutagenesis followed by antimetabolite selection	11.0	1.9	(33) Chapter 2
Increasing tyrosine availability	Targeted interruption of the gene *hpd*	13.4	2.4	Chapter 3
Improving *p*-hydroxybenzoate export	Overexpression of the genes encoding a multidrug MFS efflux transporter (PP1271-PP1273)	13.4	2.4	Chapter 3
Establishing efficient xylose utilization	Introduction of the genes *xylABFGH* followed by laboratory evolution on xylose as substrate	17.5	3.0	Chapter 4
Increasing pentose phosphate pathway activity	Co-feeding xylose and glucose	7.9[b]	0.7[b]	Chapter 4
	Co-feeding xylose and glycerol	16.3[c]	1.6[c]	Chapter 4
Increasing final *p*-hydroxybenzoate concentration	High cell density fed-batch cultivation	5.9[d]	38.4[d]	Chapter 5
Producing *p*-hydroxybenzoate from an industrial substrate	Crude glycerol as substrate	6.6[d]	43.5[d]	Chapter 5

[a] in shake flask cultivations on 120 mCM glucose unless stated otherwise

[b] In chemostat cultivations on 60 mCM xylose-glucose mixtures (100% glucose Y_{ps} = 4.9 Cmol%)

[c] In chemostat cultivations on 60 mCM xylose-glycerol mixtures (100% glycerol Y_{ps} = 8.0 Cmol%)

[d] In fed-batch cultivation on (crude) glycerol as substrate

demonstrated for *Escherichia coli*, the flux through this pathway can be optimized, *e.g.* by overexpressing tyrosine biosynthetic genes and alleviating feed-back inhibition (for reviews see (4, 7, 9)). The utility of the latter approach for *P. putida* S12 is questionable, however, since no accumulation of aromatic intermediates was observed. Another possibility for strain improvement is preventing the yield loss of approximately 5 % as a result of the conversion of phenylalanine into *t*-cinnamate by the enzyme Pal/Tal. This problem can be circumvented by constructing a phenylalanine auxotrophic mutant as described by Nijkamp et al. (22) or alternatively, by replacing the Pal/Tal enzyme by a specific tyrosine ammonia lyase (Tal; (16, 34)).

In addition to strain engineering, the production parameters can be enhanced by changing the carbon source and cultivation conditions. As demonstrated in Chapters 4 and 5, the carbon source present in the feed can positively affect the product-to-substrate yield (Table 1). The maximum product-to-substrate yield was achieved on a feed containing both glycerol and xylose. It is therefore expected that by applying this co-feeding strategy in fed-batch mode, the final *p*-hydroxybenzoate concentration can be further improved. In carbon limiting chemostat cultivations glycerol and xylose were simultaneously utilized (Chapter 4). However, a diauxic shift may occur in the presence of excess carbon as demonstrated for a mixture of xylose and glucose (19). Since the occurrence of a diauxic shift will corrupt the benefits of co-utilization, the possible substrate preferences have to be investigated.

Formation of *p*-hydroxybenzoate and cell growth is linked in *P. putida* S12. Therefore, the product titers could be improved roughly by a factor 25 by performing a high-cell density cultivation (Table 1). The maximum biomass concentration obtained in this thesis (60 g l^{-1}) is around 2 to 3-fold lower than reported values for other *P. putida* strains (13, 18). The fermentation conditions were not completely optimized for *P. putida* S12 in this thesis, so very likely also for engineered *P. putida* S12 strains higher biomass and therefore product titers can be achieved. Regrettably, the product-to-substrate yield drops at high cell density and *p*-hydroxybenzoate concentration (Chapter 5), which results in a lower overall productivity of the process. In addition to increased maintenance requirements, a plausible explanation for this phenomenon is the toxicity of *p*-hydroxybenzoate. As shown in Chapter 2, the growth rate of *P. putida* S12 is affected upon the addition of 7 g l^{-1} *p*-hydroxybenzoate. To alleviate product toxicity, *in-situ* product recovery (ISPR) can be applied as demonstrated in Chapter 6. To reduce the concentration *p*-hydroxybenzoate in the fermentation broth, ISPR techniques such as the addition of an ion exchange resin (12) or crystallization of the produced *p*-hydroxybenzoate (Jan Harm Urbanus, TNO I&T, the Netherlands, pers. communication) can presumably be applied successfully.

Is *P. putida* S12 a preferred host for the biological production of aromatics?

Several processes and microbial hosts have been applied for the biological production of *p*-hydroxybenzoate and *p*-hydroxystyrene. For example, *P. putida* strains have been employed for the production of these aromatic compounds from a renewable substrate, *i.e.* bioconversion (this thesis), as well as for the biotransformation of toluene into *p*-hydroxybenzoate (20, 21, 27). Furthermore, the production of *p*-hydroxybenzoate and *p*-hydroxystyrene by means of bioconversion has been reported for engineered *Escherichia coli* strains (1-3, 26). Obviously the production characteristics are of crucial importance for economic feasibility of the (semi) bio-based production process.

The lowest *p*-hydroxybenzoate production characteristics were reported for the biotransformation process from toluene. A derivative of the non-solvent-tolerant *P. putida* KT2440 strain achieved a final *p*-hydroxybenzoate concentration of 0.9 mM in fed-batch cultivation on glutamate as carbon source (21). Improved production was achieved using resting cells of an engineered strain of solvent-tolerant *P. putida* DOT-T1E (27). Nevertheless, the conversion rate of toluene into *p*-hydroxybenzoate (0.4 mM h^{-1}) and the final *p*-hydroxybenzoate concentration (10.5 mM; (27)) were lower than the maximum values reported in this thesis for the conversion of a renewable substrate into *p*-hydroxybenzoate (1.7 mM h^{-1} and 44 mM, respectively; Chapter 5).

The highest maximum *p*-hydroxybenzoate concentration (87 mM with a Y_{ps} of 15 Cmol%) was obtained from glucose by engineered *E. coli* cells (2) in which the production of *p*-hydroxybenzoate was established via the intermediate chorismate, by over-expressing chorismate lyase (UbiC; (1, 2)). The resulting strain, however, was multi-auxotrophic for the aromatic amino acids (2, 6) which severely limits its utility for industrial applications. Furthermore, production in *E. coli* was limited by the toxicity of *p*-hydroxybenzoate to the cells, since a *p*-hydroxybenzoate concentration above 72 mM adversely affected the microbial growth and metabolism of *E. coli* (2). As demonstrated in Chapter 2, *P. putida* S12 can tolerate higher concentrations of *p*-hydroxybenzoate in the medium broth than *E. coli*. Nevertheless, the maximum *p*-hydroxybenzoate concentration and the corresponding Y_{ps} achieved in fed-batch cultivation by an engineered *P. putida* S12 strain were 1.8-fold lower than reported for *E. coli* (Table 1). As is evident from Chapter 4, the product-to-substrate yield for engineered *P. putida* S12 can approximately be doubled by co-feeding xylose and glycerol. Thus, it may be expected that at least comparable production parameters can be obtained for *P. putida* S12 and *E. coli* in fed-batch cultivation.

The aromatic compound *p*-hydroxystyrene is far more toxic to the cells than *p*-hydroxybenzoate (Chapter 2 and 6). Due to its inherent solvent tolerance, *P. putida* S12 can tolerate both a higher *p*-hydroxystyrene concentration in the fermentation broth and a second phase of a more efficient extractant compared to *E. coli*. As a result, the production of *p*-hydroxystyrene from glucose by engineered *P. putida* S12 was higher by a factor 10 compared to the *E. coli* system in a two-phase water-organic solvent fed-batch cultivation. To alleviate product toxicity in *E. coli*, Sariaslani (29) divided the *p*-hydroxystyrene production process in three stages: production of tyrosine from glucose by *E. coli*, conversion of tyrosine into *p*-coumarate by immobilized Pal-overexpressing *E. coli* cells, and chemical decarboxylation of *p*-coumarate into *p*-hydroxystyrene.

As is evident from these production characteristics, the solvent-tolerant features of *P. putida* S12 play an important role when the product concentration in the cultivation broth reaches levels that are toxic for the cells. Especially for highly toxic compounds like *p*-hydroxystyrene, employing a solvent-tolerant strain as a production host presents a genuine benefit.

Renewable feedstock as substrate for the productin of aromatics by *P. putida* S12

Since the economic viability of biological production of chemicals on a large scale is mainly dependent on the feedstock used (24), it is of great importance to investigate the fermentability of cheap second generation renewable resources by *P. putida* S12.

Lignocellulosic hydrolysate, which is currently considered the main renewable feedstock, consists for a large part of glucose (approx. 50 %) and xylose (approx. 20%; (17)). For efficient conversion of hydrolysate into aromatics, all sugars should be utilized. Since wild type *P. putida* S12 can only utilize C_6 sugars such as glucose and fructose, an effort was made in Chapter 4 to engineer the *p*-hydroxybenzoate producing strain for xylose utilization. In addition to improved carbon efficiency, co-utilization of glucose and xylose resulted in a 1.5-fold increased aromatics production. Thus, instead of posing a disadvantage, the heterogenic nature of hydrolysate is turned into an advantage for aromatics production. Although we constructed an aromatics producing *P. putida* S12 strain that was capable of converting most of the sugars present in hydrolysate, the use of hydrolysate can be hindered by the presence of fermentation inhibitors like furanic aldehydes, organic acids and aromatic compounds. Koopman *et al* (15) demonstrated that *P. putida* S12 can degrade furanic compounds by introducing the furfural and 5-(hydroxymethyl)furfural degradation pathway of *Cupriavidus basilensis*. Thus, by introducing these genes in the production host, the furanic compounds may be utilized and accumulation of these inhibitors in the fermentation broth can be overcome. Furthermore, most of the aromatic compounds present in lignocellulosic hydrolysate (14) can be utilized by *P. putida* S12. Some of these, such as *p*-coumarate, *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde, can even be directly converted into *p*-hydroxybenzoate. To evaluate whether the strain engineering steps described above actually improve the utility of lignocellulosic hydrolysate for aromatics production by *P. putida* S12, fed-batch cultivations on raw hydrolysate should be performed.

In Chapter 5, fermentability of crude glycerol obtained as main by-product of biodiesel formation from vegetable oil, was evaluated for *P. putida* S12. Whereas for most micro-organisms the impurities present in crude glycerol strongly interfered with the fermentation process (10, 25, 28), *P. putida* S12 performed even better on crude glycerol than on purified glycerol in terms of growth and *p*-hydroxybenzoate production. The intrinsic robustness of *P. putida* S12 expectedly played a role in the tolerance to crude glycerol. Additionally, the ability to utilize long chain fatty acids (8, 30), present as impurities in crude glycerol (31), most likely contributed to the observed better performance.

As discussed, the production characteristics of *P. putida* S12pal_xylB7 on glycerol can be improved by adding xylose to the glycerol feed (Chapter 4). Thus, the product-to-substrate yield and the production rate reported for crude glycerol as substrate (Table 1 and Chapter 5) can be further improved by employing the *p*-hydroxybenzoate producing strain *P. putida* S12pal_xylB7 on a mixture of crude glycerol and xylose. To minimize the addition of the usually more expensive carbon source xylose to the cheap crude glycerol, the optimal ratio of glycerol and xylose for *p*-hydroxybenzoate production needs to be addressed. Based on the results obtained in Chapter 4, the optimal fraction of xylose will below 25 Cmol %.

Concluding remarks

The depletion of fossil resources and concerns about global warming necessitate investigation of sustainable alternatives for the petroleum-based production of organic chemicals. The work laid down in this thesis has demonstrated that industrially relevant, but toxic aromatic compounds can efficiently be produced from renewable substrates by employing the solvent-tolerant *P. putida* S12 strain as a production host. Although several optimization steps have been described in this thesis, a number of challenges still need to be addressed. These include the improvement of the productivity, efficient product recovery and scaling up of the production process. By combining the knowledge of microbiology, genetic engineering, organic chemistry and process engineering, sustainable bio-based production processes for various organic chemicals will become a reality.

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Summary
Samenvatting

Summary

To replace environmentally unfriendly petrochemical production processes, the demand for bio-based production of organic chemicals is increasing. This thesis focuses on the biological production of hydroxylated aromatics from renewable substrates by engineered *P. putida* S12 including several cases of strain improvement.

Chapter 2 describes the construction of a *P. putida* S12 strain that produces *p*-hydroxybenzoate via the aromatic amino acid tyrosine. Previous research on biosynthesis of aromatic compounds has culminated in the construction of *P. putida* S12_427. This strain, which has an optimized carbon flux towards tyrosine, was employed as platform host for aromatics production. By introducing the heterologous gene *pal/tal* (encoding phenylalanine/tyrosine ammonia lyase; Pal/Tal) the conversion of tyrosine into *p*-coumarate was established, which compound is further converted into *p*-hydroxybenzoate by endogenous enzymes. The degradation of *p*-hydroxybenzoate was prevented by inactivating the gene *pobA*, which encodes hydroxybenzoate hydroxylase. The resulting strain, *P. putida* S12palB1, accumulated *p*-hydroxybenzoate at a yield of 11 Cmol % on glucose or glycerol in shake flask cultivations. A glycerol-limited fed-batch cultivation was performed to increase the product titers, yielding a final *p*-hydroxybenzoate concentration of 12.9 mM (1.8 g l^{-1}) with a product-to-substrate yield of 8.5 Cmol %. Tyrosine availability was identified as the main bottleneck for *p*-hydroxybenzoate production in glycerol-limited chemostat cultivations.

Since the enhanced flux towards *p*-hydroxybenzoate in *P. putida* S12palB1 mainly originated from a random mutagenesis approach, multiple system-wide changes were expected. Therefore, a chemostat-based comparative transcriptomics and proteomics analysis was performed as described in **Chapter 3,** to gain insight into the genetic background of the enhanced strain performance. The overall expression differences between parent strain and *P. putida* S12palB1 confirmed the system-wide changes effectuated by the strain improvement procedure. The higher net metabolic flux towards *p*-hydroxybenzoate was reflected in the upregulation of genes involved in tyrosine biosynthesis and the conversion of *p*-coumarate into *p*-hydroxybenzoate. Notably, on glucose some of the *p*-hydroxybenzoate biosynthetic genes were upregulated to a higher extent than in the glycerol-grown chemostats, while the *p*-hydroxybenzoate accumulation was not affected by the carbon source applied. Furthermore, a multidrug efflux transporter (PP1271-PP1273) was identified that may have a major role in *p*-hydroxybenzoate export. The 2.8-fold upregulation of *hpd* (encoding 4-hydroxyphenylpyruvate dioxygenase; first enzyme of tyrosine degradation via the homogentisate pathway) on glucose suggested that part of the tyrosine was directed away from *p*-hydroxybenzoate biosynthesis. This was confirmed by a 22-% increase of the product-to-substrate yield upon eliminating *hpd*. Although the *hpd* gene was not significantly differentially expressed on glycerol (1.5-fold), *hpd* deletion resulted in a 21 % improved *p*-hydroxybenzoate yield also on glycerol.

The key precursors for the production of aromatic compounds, phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), are withdrawn from the lower glycolysis, respectively, the pentose phosphate (PP) pathway. Since the flux through the PP pathway is typically low in *P. putida* S12, the availability of E4P may be a bottleneck for *p*-hydroxybenzoate

production. In **Chapter 4**, it was attempted to increase the availability of E4P by co-feeding xylose directly into the PP pathway. To this end, the genes *xylABFGH* (encoding xylose isomerase, xylulokinase and a high affinity xylose transporter) were introduced in the *p*-hydroxybenzoate producing strain to establish growth on xylose via the PP pathway. Growth performance on xylose was improved by an evolutionary selection approach. Surprisingly, this also resulted in a 25-% improvement of *p*-hydroxybenzoate production on either glycerol or glucose, indicating that the resulting strain, *P. putida* S12pal_xylB7, had an intrinsically elevated PP pathway activity. Chemostat experiments demonstrated that co-feeding of xylose (replacing part of the glucose or glycerol in the feed) considerably increased the production efficiency. On glucose, co-feeding of xylose improved the product-to-substrate yield by a factor 1.5 to 7.9 Cmol %, while on glycerol the product-to-substrate yield doubled to 16.3 Cmol %. Interestingly, product formation was not further improved by replacing more than 25 % of the carbon feed with xylose. This suggested that the availability of E4P was no longer the limiting step in *p*-hydroxybenzoate biosynthesis. The ability of co-utilizing xylose and glucose and the resultant improved production parameters implicated that lignocellulosic feedstock, containing around 20 % xylose and 50 % glucose, is a very suitable substrate for the production of aromatic compounds by engineered *P. putida* S12.

In **Chapter 5** the utilization of the industrial grade renewable feedstock crude glycerol was evaluated. In high-cell density fed-batch fermentations, *P. putida* S12 strains performed consistently better on crude glycerol than on purified glycerol, as shown by the higher biomass-to-substrate yield, maximum biomass production rate and substrate uptake rate. Moreover, the production of *p*-hydroxybenzoate by an engineered *P. putida* S12 strain was more efficient on crude glycerol. On crude glycerol a maximum *p*-hydroxybenzoate concentration of 43.5 mM (6.0 g l^{-1}) was obtained with a product-to-substrate yield of 6.6 Cmol %, compared to 38.4 mM (5.3 g l^{-1}) and 5.9 Cmol % on purified glycerol. In contrast, *E. coli* DH5α showed a decreased biomass-to-substrate yield and growth rate on crude glycerol compared to purified glycerol, and increased acetate formation (11.5 and 16.2 g l^{-1} on purified and crude glycerol respectively).

The majority of the optimization approaches described in this thesis for *p*-hydroxybenzoate production can be applied for efficient production of other tyrosine derived compounds. This is exemplified in **Chapter 6**, where the production of the value-added compound *p*-hydroxystyrene from glucose is described. Production of *p*-hydroxystyrene was established by introducing the genes *pal/tal* and *pdc*, encoding phenylalanine/tyrosine ammonia lyase and *p*-coumaric acid decarboxylase respectively, into the tyrosine overproducing strain *P. putida* S12_427. These enzymes allow the conversion of the central metabolite tyrosine into *p*-hydroxystyrene, via *p*-coumarate. Degradation of the *p*-coumarate intermediate was prevented by inactivating the *fcs* gene encoding feruloyl-CoA synthetase. In fed-batch cultivation on glucose a maximum *p*-hydroxystyrene concentration of 4.5 mM was obtained with a yield of 6.7 Cmol %. At this *p*-hydroxystyrene 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 approach resulted in a final *p*-hydroxystyrene concentration of 147 mM (17.6 g l^{-1}) in the decanol phase, with a twofold increase of the maximum volumetric productivity (0.75 mM h^{-1}) and a fourfold higher total concentration (21 mM).

The work described in this thesis illustrates how hydroxylated aromatics can be efficiently produced by engineered *P. putida* S12 strains. Several strategies for analysis and optimization were performed to improve the aromatics production and understand the background and impact of the improvements. The biological production of various hydroxylated aromatics was established and largely improved. Hence, this work presents an important contribution to our efforts to replace petrochemical production by bio-based processes.

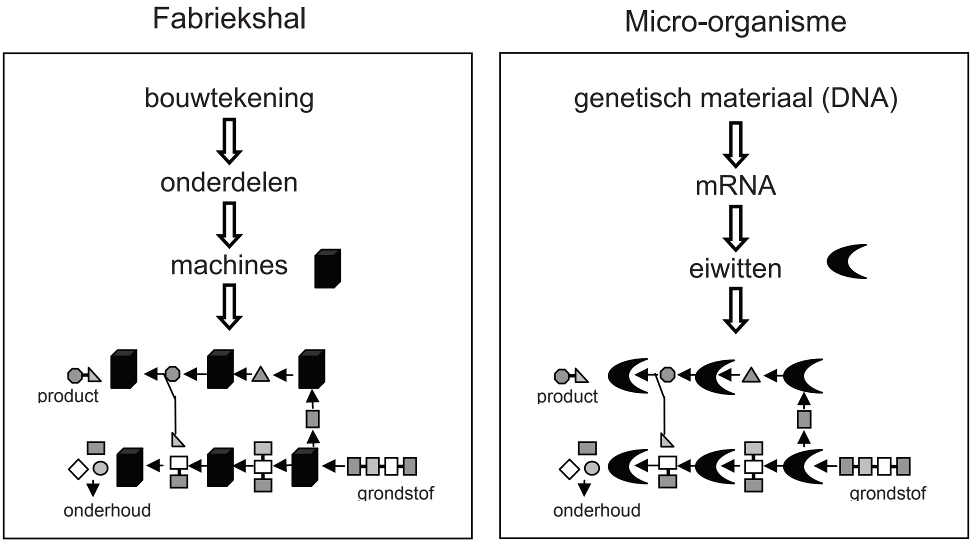
Suzanne Verhoef

Samenvatting voor de niet-ingewijden

Veel van de dagelijks gebruikte producten bevatten organische verbindingen (koolstof bevattende moleculen) die hoofdzakelijk worden geproduceerd vanuit fossiele grondstoffen zoals olie. Deze fossiele grondstoffen beginnen echter uitgeput te raken en de petrochemische productieprocessen zijn veelal milieuvervuilend. Er is daardoor een toenemende vraag naar geschikte duurzame alternatieve productieprocessen. Om tot een duurzaam productieproces te komen, is het van belang dat de fossiele grondstoffen vervangen worden door duurzame hernieuwbare grondstoffen. Voorbeelden van deze hernieuwbare grondstoffen zijn suikers (o.a. glucose en xylose) en glycerol. De suikers kunnen verkregen worden door plantaardig materiaal zoals stengels, bladeren en boomstammen te bewerken. Glycerol is een bijproduct dat vrijkomt tijdens de productie van biodiesel uit plantaardige vetten.

Micro-organismen zoals schimmels, gisten en bacteriën zijn in staat om deze hernieuwbare grondstoffen om te zetten in allerlei organische verbindingen. Een micro-organisme kan ook wel gezien worden als een 'kleine fabriekshal' waarin allemaal verschillende machines staan die eiwitten genoemd worden. De eerste machine zet de grondstof om in een bepaalde bouwsteen en door veel verschillende machines achter elkaar te zetten, kan er iedere keer een andere bouwsteen gemaakt worden totdat je bij het gewenste product bent aangekomen (Figuur 1). Door bepaalde machines uit te schakelen of juist meerdere van dezelfde machine in de fabriekshal te plaatsen, is het mogelijk om op een efficiëntere manier de grondstof in het product om te zetten.

Er is echter maar een beperkte hoeveelheid van de grondstof beschikbaar om het gewenste product te maken, omdat micro-organismen een deel van de grondstof gebruiken om de



Figuur 1. Versimpelde weergave van de overeenkomsten tussen een 'fabriekshal' en een micro-organisme. Door machines c.q. eiwitten kan de grondstof, via allerlei bouwstenen, omgezet worden naar het gewenste product.

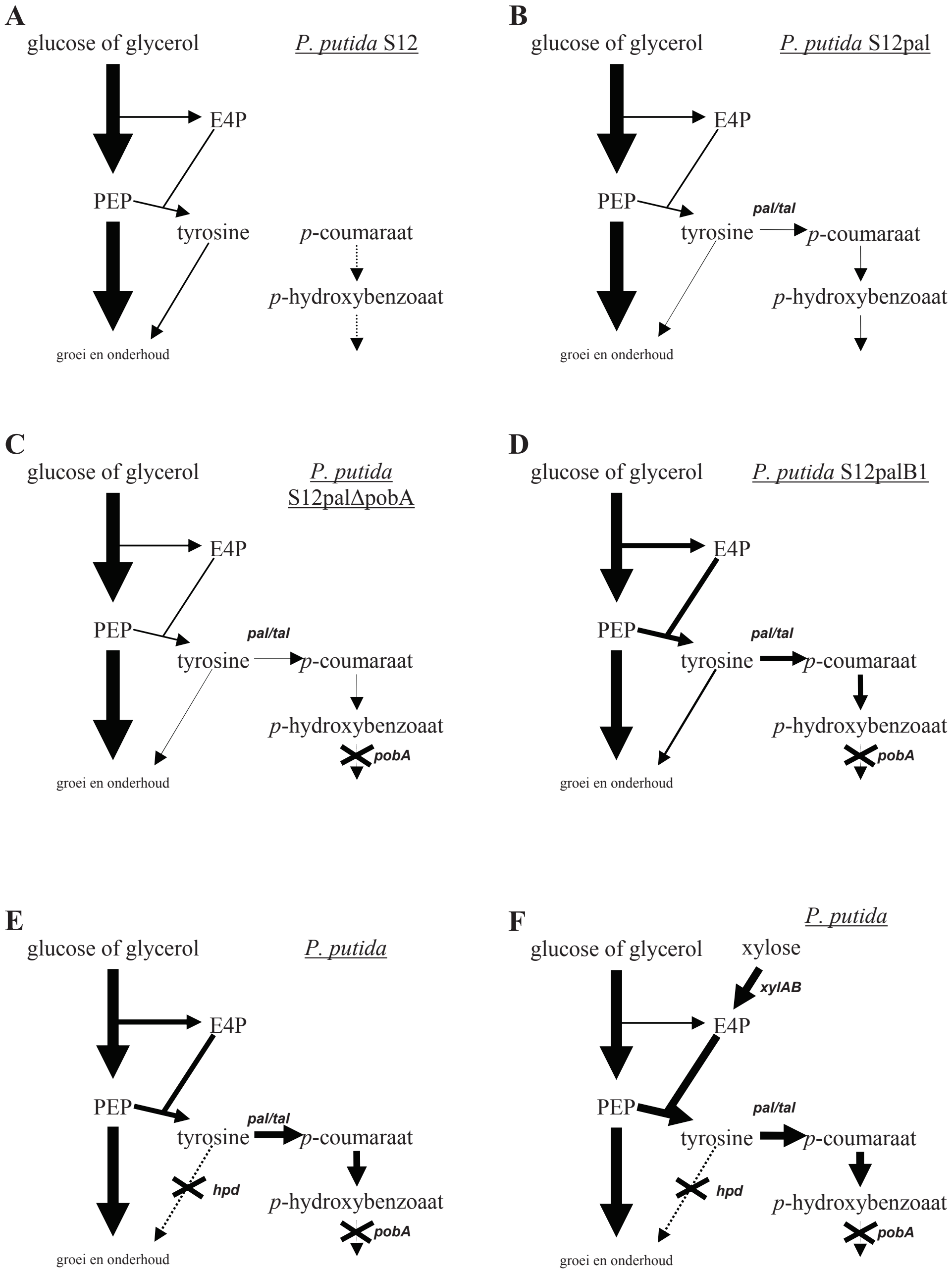
‘fabriekshal’ te onderhouden (bijvoorbeeld voor verlichting, verwarming en repareren van machines). Tevens heeft het micro-organisme grondstof nodig om zichzelf de vermenigvuldigen.

Het genetische materiaal (DNA) van het micro-organisme, ook wel de bouwtekening van de fabriekshal, bepaalt welke machines er in de fabriek komen te staan (Figuur 1). In het DNA bevinden zich allemaal kleine stukjes (genen) die ieder voor zich een bouwtekening zijn voor een bepaald eiwit. Door een gen af te lezen wordt er mRNA gemaakt dat omgezet kan worden in een eiwit (Figuur 1). Een complex regelmechanisme bepaalt hoeveel en welke mRNA’s en eiwitten er in het micro-organisme aanwezig zijn.

In dit proefschrift is beschreven hoe je de ‘bouwtekening’ van een bacterie zo kunt veranderen dat zij efficiënt aromatische verbindingen kan produceren vanuit een hernieuwbare grondstof. De aromatische verbindingen die beschreven zijn in deze studie, *p*-hydroxybenzoaat en *p*-hydroxystyreen, worden onder andere gebruikt voor de productie van plastics en als conserveermiddel in de vorm van parabenen in cosmetica. Deze aromatische verbindingen zijn vaak extreem giftig voor micro-organismen. We hebben daarom een bacterie geselecteerd die verschillende afweersystemen heeft tegen dit type giftige stoffen. De bacterie die we in dit werk gebruiken als ‘fabriekshal’ is *Pseudomonas putida* S12.

De constructie van een bacterie die *p*-hydroxybenzoaat produceert

In hoofdstuk 2 is beschreven hoe de bacterie *P. putida* S12 is aangepast zodat vanuit een hernieuwbare grondstof de aromatische verbinding *p*-hydroxybenzoaat gemaakt kan worden. Figuur 2 geeft een sterk vereenvoudigde weergave van de ‘fabriekshal’ van *P. putida* S12 weer. De aanpassingen die gedaan zijn om tot een efficiënte productie van *p*-hydroxybenzoaat te komen zijn weergegeven in de verschillende subfiguren. De dikte van de pijlen geeft aan hoeveel grondstof er van de ene machinekamer naar de andere loopt. Zoals in figuur 2A te zien is, mist *P. putida* S12 het eiwit (machine) dat zorgt voor de omzetting van tyrosine naar *p*-coumaraat. Door het gen (stukje bouwtekening) *pal/tal* toe te voegen aan het genetische materiaal van *P. putida* S12 wordt het eiwit phenylalanine/tyrosine ammonia lyase gemaakt Dit eiwit zorgt ervoor dat tyrosine omgezet kan worden in *p*-coumaraat (figuur 2B). Omdat *P. putida* S12 een eiwit heeft die *p*-hydroxybenzoaat om kan zetten in een andere bouwsteen, is het gen dat zorgt voor dat bepaalde eiwit (PobA) verwijderd uit het genetische materiaal van *P. putida* S12 (figuur 2B). Deze aanpassingen resulteerde echter in lage productie van *p*-hydroxybenzoaat, omdat de grondstofstroom naar het product erg laag was. De grondstofstroom kan verhoogd worden door meer van bepaalde machines in de fabriekshal te plaatsen of door de machines een beetje aan te passen. Er is een voorgaand onderzoek geweest waarin dit al onderzocht en uitgevoerd was voor de machines van de grondstof naar de bouwsteen tyrosine. Omdat het verbeteren van machines een tijdrovend proces is, hebben we in dit onderzoek voor een deel gebruik gemaakt van de resultaten uit voorgaand onderzoek. In ons onderzoek hebben we de machine pal/tal toegevoegd en de machine PobA uitgeschakeld. Het resultaat is de bacterie *P. putida* S12palB1 (figuur 2C). Zoals aan de dikte van de pijl te zien is, stroomt er meer grondstof naar het product in deze bacterie. *P. putida* S12palB1 is in staat om de grondstoffen glucose of glycerol om te zetten in *p*-hydroxybenzoaat met een efficiëntie van ongeveer 11 Cmol % (percentage van de hoeveelheid koolstof). Dit komt overeen met de productie van 0.07 gram *p*-hydroxybenzoaat vanuit 1 gram glucose.



Figuur 2. Sterk vereenvoudigde weergave van de grondstof stroom naar product en ‘groei en onderhoud’ voor verschillende (aangepaste) P. putida *S12 bacteriën. De dikte van de pijl geeft de hoeveel van de grondstof aan die van de ene naar de andere bouwsteen gaat. Een gestippelde lijn betekent dat dit eiwit (machine) niet werkt. Een gestreepte lijn geeft aan dat het eiwit er wel is maar er geen bouwstenen aanwezig zijn die door het eiwit omgezet kunnen worden.*

Elke bacterie kan maar een beperkte hoeveelheid *p*-hydroxybenzoaat maken. Dus voor veel *p*-hydroxybenzoaat heb je veel bacteriën nodig. Een deel van de grondstof wordt door de bacterie gebruikt om zich te vermeerderen (figuur 2). Als er veel grondstof aanwezig is, zullen er dus veel bacteriën gevormd kunnen worden die allemaal *p*-hydroxybenzoaat maken. Uiteindelijk is er door *P. putida* S12palB1 vanuit ongeveer 32 gram glycerol 13 gram bacteriën en 1.8 gram *p*-hydroxybenzoaat per liter gemaakt. Dit komt overeen met een efficiëntie van 8.5 C mol %.

Analyse en verbetering van de *p*-hydroxybenzoaat producerende bacterie *P. putida* S12palB1

Om te achterhalen wat er precies voor heeft gezorgd dat *P. putida* S12palB1 zoveel *p*-hydroxybenzoaat kan maken vanuit een hernieuwbare grondstof, is er in hoofdstuk 3 gekeken naar de hoeveelheid die van elk individueel mRNA (bouwonderdeel) en eiwit (machine) aanwezig is in *P. putida* S12palB1 (figuur 2C). Door dit te vergelijken met de basis bacterie *P. putida* S12 (figuur 2A), konden we zien welke mRNA's en eiwitten er veranderd waren. Vaak geeft de hoeveelheid van mRNA en eiwit aan hoe productief de machine is. Dus hoe meer mRNA en/of eiwit hoe meer grondstof er door deze machine loopt.

Uit de vergelijking kwam naar voren dat er in *P. putida* S12palB1 meer mRNA's aanwezig waren, die te maken hebben met de vorming van *p*-hydroxybenzoaat vanuit de bouwstenen PEP en E4P (Figuur 2). Bovendien werd er nog een hoger mRNA niveau gevonden van het gen *hpd*. Het bijbehorende eiwit zorgde ervoor dat tyrosine omgezet kon worden in andere bouwstenen, waardoor er minder tyrosine beschikbaar was voor de productie van *p*-hydroxybenzoaat (het lekt weg door een 'zijbuis'). Door het gen *hpd* te verwijderen uit het genetische materiaal van *P. putida* S12palB1 was het mogelijk om meer grondstof naar *p*-hydroxybenzoaat te laten stromen (figuur 2D). Uiteindelijk werd de grondstoffen stroom naar *p*-hydroxybenzoaat met 22 % verhoogd tot een efficiëntie van 13.4 Cmol % (0.09 gram *p*-hydroxybenzoaat uit 1 gram glucose).

Zoals te zien is in figuur 2, zijn de bouwstenen PEP en E4P nodig om *p*-hydroxybenzoaat te maken. Voor elke *p*-hydroxybenzoaat die gemaakt wordt zijn er 2 stuks van PEP nodig en 1 van E4P. In *P. putida* S12 is de stroom glucose of glycerol naar E4P erg laag en vermoedelijk is dit het knelpunt voor de productie van *p*-hydroxybenzoaat. Door nu xylose als grondstof aan te bieden, kan de stroom naar E4P verhoogd worden. *P. putida* S12palB1 mist echter de eiwitten xylose isomerase en xylulokinase die ervoor zorgen dat xylose gebruikt kan worden (figuur 2A). De genen *xylA* en *xylB* werden toegevoegd aan het genetische materiaal van *P. putida* S12palB1, zodat het mogelijk was om xylose als grondstof te gebruiken (figuur 2E).

Door een mengsel van de grondstoffen glucose en xylose aan te bieden aan *P. putida* S12pal_xylB7 (Figuur 2E), werd de productie efficiëntie met een factor 1.8 naar 7.9 Cmol % verhoogd ten opzichte van afzonderlijk toevoegen van glucose of xylose. Op een mengsel van glycerol en xylose werd de hoogste efficiëntie behaald, namelijk van 16.3 Cmol %. Dit komt neer op de productie van 0.11 gram *p*-hydroxybenzoaat vanuit 1 gram grondstof.

De productie van *p*-hydroxybenzoaat vanuit een industriële grondstof

Om tot een economisch haalbare productie te komen, is het van groot belang dat de grondstof niet te duur is. In hoofdstuk 5 is bekeken of de goedkope ruwe glycerol te gebruiken is als grondstof voor de productie van *p*-hydroxybenzoaat. In de ruwe glycerol zitten verontreinigingen waarvan de meeste micro-organismen last hebben. Deze verontreinigingen zorgen ervoor dat de micro-organismen stoppen met groeien en produceren. *P. putida* S12 heeft, waarschijnlijk door zijn afweersystemen tegen toxische stoffen, geen last van deze verontreinigingen. Voor *P. putida* S12 werd er zelfs een betere prestatie gemeten door ruwe glycerol in plaats van schone glycerol aan te bieden als grondstof. Uiteindelijk werd er 6 gram *p*-hydroxybenzoaat per liter geproduceerd vanuit ruwe glycerol.

De constructie van een bacterie die *p*-hydroxystyreen produceert

De technologieën die hierboven beschreven zijn kunnen ook toegepast worden voor de productie van andere gelijksoortige producten. In hoofdstuk 6 is er gekeken naar de productie van *p*-hydroxystyreen. Dit is een aromatische verbinding die erg giftig is voor micro-organismen. *p*-hydroxystyreen kan, net als *p*-hydroxybenzoaat, gemaakt worden uit de bouwsteen tyrosine. Daarom is ook hier uitgegaan van de *P. putida* S12 bacterie die veel tyrosine maakt (Figuur 2C). Omdat *P. putida* S12 de eiwitten (machines) mist die tyrosine omzetten in *p*-hydroxystyreen, zijn de genen (bouwtekeningen) *pal/tal* en *pdc* aan het genetische materiaal toegevoegd. Om geen verlies te krijgen door de afbraak van *p*-coumaraat is het gen *fcs* uit het genetische materiaal verwijderd (figuur 2F).
Omdat *p*-hydroxystyreen heel erg giftig is voor *P. putida* S12 kon er maar 0.54 gram *p*-hydroxystyreen per liter gemaakt worden voordat de bacteriën dood gingen. In hoofdstuk 6 hebben we een oplossing bedacht om de *p*-hydroxystyreen, die gevormd wordt door de bacteriën, gelijk te verwijderen uit de buurt van de bacteriën zodat ze niet doodgaan. Door het organische oplosmiddel decanol toe te voegen lost de *p*-hydroxystyreen op in de decanol. Hierdoor bleef de concentratie *p*-hydroxystyreen in de omgeving van de bacteriën laag en gingen de bacteriën niet dood. Uiteindelijk werd er 2.20 gram *p*-hydroxystyrene gemaakt, wat 4 keer zoveel is als zonder de toevoeging van decanol.

Conclusie

In dit proefschrift is aangetoond dat de bacterie *P. putida* S12 zo aangepast kan worden dat vanuit hernieuwbare grondstoffen zoals glucose, xylose en glycerol de aromatische verbindingen *p*-hydroxybenzoaat en *p*-hydroxystyreen gemaakt kunnen worden. In de verschillende hoofdstukken is beschreven hoe de productie efficiëntie verhoogd kon worden tot maximaal 0.11 gram *p*-hydroxybenzoaat vanuit 1 gram grondstof. Het werk uitgevoerd in dit proefschrift is een belangrijke bijdrage in het vervangen van petrochemische productie door duurzame biologische productie van organische verbindingen zoals *p*-hydroxybenzoaat en *p*-hydroxystyreen.

Suzanne Verhoef

Curriculum vitae
Publications
Dankwoord

Curriculum vitae

Suzanne Verhoef werd geboren op 21 augustus 1980 in Oldebroek. Zij behaalde haar VWO diploma in 1998 aan het Carolus Clusius College te Zwolle. Aansluitend volgde ze de studie Bioprocestechnologie aan de Wageningen Universiteit. Tijdens deze opleiding werden er afstudeervakken gedaan bij het Laboratorium voor Microbiologie van Wageningen Universiteit en bij het Instituut voor Agrotechnologisch Onderzoek (ATO, nu AFSG) te Wageningen. Ten slotte heeft zij stage gelopen in Nieuw Zeeland bij het Institute of Technology and Engineering van Massey University te Palmerston North. Deze studie werd met succes afgerond in 2004. In maart 2005 is zij begonnen als Assistent in Opleiding bij de Technische Universiteit van Delft. Van maart 2005 tot en met december 2006 werd het onderzoek uitgevoerd bij het voormalige instituut TNO-MEP in Apeldoorn onder begeleiding van Jan de Bont en Jan Wery. Vanaf 1 januari 2007 is de onderzoeksgroep (nu afdeling Bioconversie in het kerngebied Kwaliteit van Leven van TNO) verhuisd naar Delft waarna de begeleiding is overgenomen door Han de Winde en Harald Ruijssenaars. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Vanaf 1 maart 2010 is Suzanne werkzaam als scientist bij Purac te Gorinchem.

Publications

***Verhoef S**, Ruijssenaars HJ, de Bont JAM, Wery J*
Bioproduction of *p*-hydroxybenzoate from renewable feedstock by solvent-tolerant *Pseudomonas putida* S12.
Journal of Biotechnology (2007) 132 49-56.

***Verhoef S**[*], Wierckx N[*], Westerhof RGM, de Winde JH, Ruijssenaars HJ*
Bioproduction of *p*-hydroxystyrene from glucose by the solvent-tolerant bacterium *Pseudomonas putida* S12 in a two-phase water-decanol fermentation.
Applied and Environmental Microbiology (2009) 75 931-936.

[*] Both authors contributed equally to this work

***Verhoef S**, Ballerstedt H, Volkers RJM, de Winde JH, Ruijssenaars HJ*
Comparative transcriptomics and proteomics of *p*-hydroxybenzoate producing *Pseudomonas putida* S12: novel responses and implications for strain improvement.
Appl Microbiol Biotechnol (2010) Accepted

***Verhoef S**, Gao N, de Winde JH, Ruijssenaars HJ*
Crude glycerol as feedstock for the production of substituted aromatics: a case for *Pseudomonas putida* S12.
Submitted

*Meijnen JP[*], **Verhoef S**[*], Briedjlal AA, de Winde JH, Ruijssenaars HJ*
Improved aromatics production by engineered *Pseudomonas putida* S12 using a mixed substrate-feeding strategy.
Submitted

[*] Both authors contributed equally to this work

***Verhoef S**, Ebert BE, Blank LM, de Winde JH and Ruijssenaars HJ*
Metabolic flux control through primary metabolism in *p*-hydroxybenzoate producing *Pseudomonas putida* S12.
Manuscript in preparation

Dankwoord

Dit is het laatste stukje dat geschreven dient te worden en dan is het klaar. Na 5 jaar is mijn boekje af! De afgelopen 5 jaar waren niet altijd gemakkelijk, maar ik kijk er met heel veel plezier op terug. Dit kwam zeker door de mensen om me heen, die ik daarom ook graag wil bedanken.

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