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The solvent-tolerant *Pseudomonas putida* S12 as host for the production of cinnamic acid from glucose

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Abstract A *Pseudomonas putida* S12 strain was constructed that efficiently produced the fine chemical cinnamic acid from glucose or glycerol via the central metabolite phenylalanine. The gene encoding phenylalanine ammonia lyase from the yeast *Rhodosporidium toruloides* was introduced. Phenylalanine availability was the main bottleneck in cinnamic acid production, which could not be overcome by the overexpressing enzymes of the phenylalanine biosynthesis pathway. A successful approach in abolishing this limitation was the generation of a bank of random mutants and selection on the toxic phenylalanine anti-metabolite *m*-fluoro-phenylalanine. Following high-throughput screening, a mutant strain was obtained that, under optimised culture conditions, accumulated over 5 mM of cinnamic acid with a yield (Cmol%) of 6.7%.

Introduction

Whole-cell biocatalysis has long been regarded as a 'green' alternative for the chemical synthesis of substituted aromatics, aldehydes, alcohols and epoxides (Parales et al. 2002; Schmid et al. 2001). Currently, the vast majority of such compounds is produced from fossil resources by production methods that often involve expensive chemical activating/protecting groups and/or large amounts of solvents.

Generally, whole-cell biocatalysis is applied in a process that involves the transformation of one preformed compound into a desired product in one or few steps. In our laboratory, whole-cell bioprocesses for the production of substituted aromatics from renewable substrates, such as sugars, are developed. The general approach hereby is to introduce one or few (heterologous) genes responsible for the conversion of central metabolites into the products of interest followed by random optimisation methods and high-throughput screening. As some substituted aromatics are very toxic to living cells, we employ the solvent-tolerant bacterium *Pseudomonas putida* S12 as the host for these processes.

P. putida S12 has an extraordinary tolerance for toxic chemicals and also a very broad biocatalytic potential (De Bont 1998; Wery and de Bont 2004). An additional advantage of the use of this strain is its ability to actively extrude a broad variety of chemicals by means of a solvent pump (Isken and de Bont 1996; Kieboom et al. 1998; Rojas et al. 2001). This mechanism not only protects the cell from toxic effects. It also serves as a driver of a biocatalytic conversion by exporting a product from the cell into the medium. Thus, also in the case of less toxic, usually more polar aromatics, *P. putida* S12 may offer better production characteristics. In this report we focus on the production of cinnamic acid from glucose via the central metabolite phenylalanine.

In nature, cinnamic acid occurs in plants as precursors of various phenylpropanoids, such as lignins, flavonoids and coumarins (Hanson and Havir 1972). Nowadays, cinnamic acid is chemically produced via a condensation reaction of acetic anhydride and benzaldehyde in the presence of sodium acetate. This process is not 'green' because the reaction occurs at high temperatures and is based on fossil resources. Methods of isolation and purification of cinnamic acid from plants are known (Benkrief et al. 1998). However, these methods are time-consuming and cumbersome, and a more facile method of production is desired for the commercial, large-scale synthesis of this aromatic acid. Cinnamic acid is very useful in anti-bacterial, flavouring and surgical applications (Burt 2004; Hoskins 1984; Miyamoto et al. 2004).

The gene encoding phenylalanine ammonia lyase (PAL, E.C. 4.3.1.5) from *Rhodosporidium toruloides* catalyses

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the deamination of phenylalanine and tyrosine to form cinnamic acid and 4-hydroxycinnamic acid, respectively (Fritz et al. 1976; Hodgins 1971). PAL homologues are found in a variety of plants, fungi and yeasts (Fritz et al. 1976; Hodgins 1971). Xiang and Moore (2002) were the first to describe the engineered biosynthesis of cinnamic acid in a bacterium by a bacterial PAL. The enzyme is used in a number of studies, to investigate its use as a possible treatment for phenylketonuria (PKU) (Sarkissian et al. 1999). The reverse reaction is used in the production of phenylalanine (El-Batal 2002; Orndorff et al. 1988; Yamada et al. 1981). Recently, the crystal structure of PAL from *R. toruloides* was elucidated (Calabrese et al. 2004).

To date, no efficient biological process for the production of cinnamic acid from glucose has been described in literature. Here we report the development of such process based on the PAL-catalysed conversion of de novo synthesised phenylalanine into cinnamic acid in the solvent tolerant host *P. putida* S12.

Materials and methods

Bacterial strains and plasmids

The strains and plasmids used in this study are shown in Table 1. *P. putida* S12 was isolated as a styrene-degrading bacterium (Hartmans et al. 1990).

Table 1 Strains and plasmids used in this study

	Characteristics	Source or reference
Strains		
P. putida S12	Wild type	Hartmans et al. (1990)
P. putida S12nc	*P. putida* S12 with plasmid pTn-1, negative control	This study
P. putida S12pal	*P. putida* S12 with plasmid pJWpalTn	This study
P. putida S12palM12	Cinnamic acid overproducing mutant of *P. putida* S12 with plasmid pJWpalTn, after NTG-mutagenesis and *m*-fluoro-phenylalanine selection	This study
Plasmids		
pGEM-T	Ap^r, *ori*ColE1, *lacZ*α, SP6, T7, *lac* promoters, direct cloning of PCR products	Promega
pT*pal*RT	pGEM-T containing the 2.2-kb *pal* gene from *R. toruloides*	This study
pTn-1	Derived from plasmid pJWB1 (Wery et al. 2001)	Unpublished data
pJWpalTn	pTn-1 containing the 2.2-kb *pal* gene from *R. toruloides*	This study

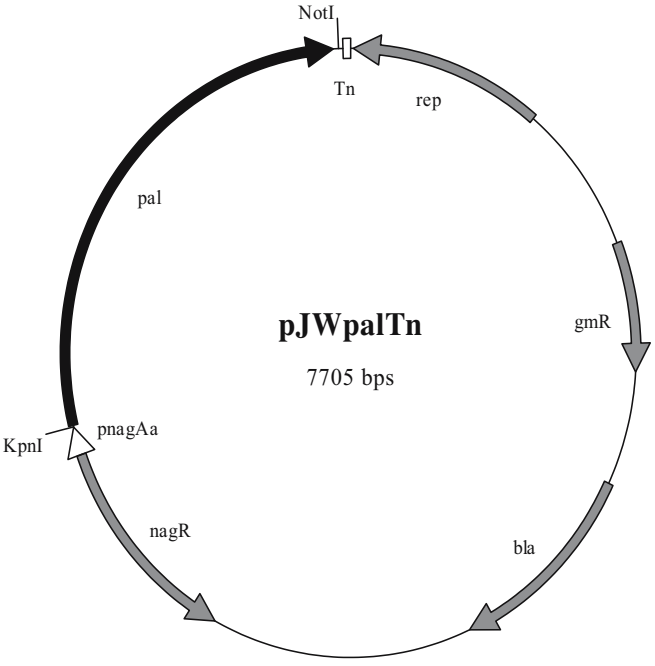

Fig. 1 Expression vector pJWpalTn. *pal* Phenylalanine ammonia lyase, *Tn* terminator, *rep* origin of replication, *gmR* gentamycin resistance gene, *bla* beta-lactamase, *nagR/pnagAa* promoter region

The expression vector pJWpalTn (Fig. 1) was constructed as described below: mRNA from *R. toruloides* (ATCC 64815) was isolated as described (Sarkissian et al. 1999). Oligonucleotides 5′-GCGGTACCATGGCACCCT CGCTCGACTCGATC-3′ (forward) and 5′-GCAAGCTT CTAAGCGAGCATCTTGAGGAGG-3′ (reverse) were designed on the published sequence of the *R. toruloides pal* mRNA (GenBank accession no. X51513) and included restriction sites for insertion in the expression vector pTn-1. pTn-1 was constructed from pJWB1 (Wery et al. 2001) by insertion of the salicylate inducible NagR/PnagAa promoter (Hüsken et al. 2001) and a transcriptional terminator at the start and end of the multiple cloning site, respectively. RT-PCR amplification was performed using the One-Step RT-PCR Kit (Qiagen) following the manufacturer's instructions. The RT-PCR product was analysed on a 0.8% agarose gel and subsequently cloned in pGEM-T (Promega) to obtain pT*pal*RT. The *pal* gene was excised from pT*pal*RT by *Kpn*I/*Not*I digestion. Ligation of *pal* into the corresponding sites of pTn-1 resulted in pJWpalTn (Fig. 1). *P. putida* S12 was transformed with pJWpalTn yielding the strain *P. putida* S12pal.

Culture conditions

The media used were Luria–Bertani broth (LB) (Sambrook et al. 1982) and a phosphate-buffered mineral medium as previously described (Hartmans et al. 1989). In mineral media, 20 mM glucose was used as the sole source of carbon (MMG), unless stated otherwise. Antibiotics were added as required to the media at the following concentrations: ampicillin, 100 μg ml^{-1}; gentamycin, 10 μg

ml^{-1}. To induce expression of *pal*, 0.1 mM of the inducer salicylate was added.

Shakeflask experiments were performed in 250-ml Erlenmeyer flasks containing 50 ml of MMG in a horizontally shaking incubator at 30°C. Cultures were inoculated to a starting OD_{600} of 0.2 with an overnight culture of uninduced cells.

Nitrogen-limited fed-batch experiments (carbon/nitrogen ratio of 20) were performed in a BioFloIIc fermentor (New Brunswick Scientific). Initial batch fermentation was started from a 50-ml inoculum of an overnight culture in MMG. The stirring speed was set to 200 rpm and pure oxygen (air with glycerol as the carbon source) was supplied at 100 ml min^{-1}. Dissolved oxygen tension was kept on 15% oxygen saturation (15% air saturation with glycerol as the carbon source) by automatic adjustment of the stirring speed. After depletion of the initial ammonium, the feed was started.

For the initial batch an adapted mineral medium (ZHDP) was used with the following composition (per litre): 25 mmol glucose or 50 mmol glycerol, 90 mg/l NH_4Cl, 5.2 g K_2HPO_4, 2.8 g $NaH_2PO_4{\cdot}H_2O$, 0.3 g $MgCl_2{\cdot}6H_2O$, 0.2 g Na_2SO_4, 10 ml trace element solution, 0.5 g yeast extract, 10 mg gentamycin, 0.1 mmol salicylate. The following composition was used for the feed (per litre): 750 mmol glucose or 1.5 mol glycerol, 4 g NH_4Cl, 3 g Na_2SO_4, 1.5 g $MgCl_2{\cdot}6H_2O$, 1.2 g $CaCl_2$, 100 ml trace element solution, 0.5 g yeast extract, 10 mg gentamycin, 0.1 mmol salicylate. The trace element solution had the following composition (per litre): 4 g EDTA, 0.2 g $ZnSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 6.5 g $FeSO_4{\cdot}7H_2O$, 0.02 g $Na_2MoO_4{\cdot}2H_2O$, 0.2 g $CuSO_4{\cdot}5H_2O$, 0.04 g $CoCl_2{\cdot}6H_2O$, 0.1 g $MnCl_2{\cdot}4H_2O$, 0.024 g H_3BO_3, 0.02 g $NiCl{\cdot}6H_2O$. Addition of feed was set at a constant rate of 35 ml h^{-1}. Medium samples (5 ml) were taken during the fermentation to determine OD_{600}, glucose, and cinnamic acid concentrations. Nitrogen limitation was confirmed by off-line analysis of medium samples via cation exchange chromatography. The pH was maintained at 7.0 with 4 N KOH.

Analytical methods

Cell densities were measured at 600 nm with a Helios Alpha spectrophotometer (Fisher Scientific). Cinnamic acid and coumaric acid concentrations were analysed by HPLC (Agilent 1,100 system) using a Zorbax 3.5 μm SB-C18 column (4.6×50 mm) with acetonitrile: NaH_2PO_4 buffer (50 mM, pH 4.5) (30:70) as an eluent (retention time coumaric acid, 0.46 min; cinnamic acid, 1.01 min). Phenylalanine, glucose and glycerol concentrations were analysed by HPLC (Waters) using an Aminex HDP-87N column with 0.01 M Na_2HPO_4 as an eluent (RT glucose, 10.6 min; glycerol, 12.9 min; phenylalanine, 14.8 min). Gluconic acid and 2-ketogluconic acid concentrations were analysed by HPLC (Waters) using an Aminex HDP-87H column with 0.008 N H_2SO_4 as an eluent (RT 2-ketogluconic acid, 8.1 min; gluconic acid, 8.9 min). NH_4^+ concentrations were determined by cation exchange chromatography (Dionex). Total protein in cell free extract was determined using the Bradford assay (Bradford 1976).

Enzymatic assay

Strain *P. putida* S12pal was grown in 100 ml of MMG with or without salicylate until the mid-exponential phase. The cells were harvested and sonicated with a Branson Sonifier (three times, 45 s) to give cell extracts. To remove cell debris, the extract was centrifuged (15 min at 16,100×*g*) and the supernatant was desalted using a PD-10 desalting column (Amersham). The reaction mixture for assaying phenylalanine ammonia lyase activity contained 1 mM phenylalanine and 25 μl of cell extract in 1 ml of 100 mM Tris buffer (pH 8.0). The PAL activity was determined at 30°C by spectrophotometrically measuring the rate of appearance of cinnamic acid at 278 nm (molar extinction coefficient is 21,000 cm^{-1}).

N-Methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) mutagenesis and selection of cinnamic acid overproducing mutants

An overnight culture of *P. putida* S12pal was mutagenised with 50 μg ml^{-1} NTG (30′ at 30°C) (Adelberg et al. 1965) and the cells were plated on minimal medium agar with 20 mM fructose and 100 μg ml^{-1}*m*-fluoro-phenylalanine (Fiske et al. 1983). A mutant bank of 2×10^4*m*-fluoro-phenylalanine resistant colonies was established using a Versarray colony arrayer and picker (Biorad). The mutant bank was duplicated in MMG with salicylate and screened for cinnamic acid overproducing mutants by measuring the OD_{278} of the culture supernatants. A selection of mutants was made and 20-ml batch cultivations were used to confirm the results.

Results

Cloning and functional expression of phenylalanine ammonia lyase in *Pseudomonas putida* S12

Phenylalanine ammonia lyase from the yeast *R. toruloides* deaminates both phenylalanine and tyrosine to cinnamic acid and 4-hydroxycinnamic acid, respectively. In the reaction with tyrosine, the enzyme's activity is designated tyrosine ammonia lyase (TAL). In *R. toruloides* the ratio of TAL to PAL activity is 0.58 (Hanson and Havir 1981).

The *pal* gene from *R. toruloides* contains six introns (Anson et al. 1987). Therefore mRNA was isolated from a culture of the yeast grown under *pal*-inducing conditions (Sarkissian et al. 1999). *pal* cDNA was synthesised by means of RT-PCR and cloned under control of the salicylate inducible promoter NagR/PnagAa (Hüsken et al. 2001) into the expression vector pTn-1. This resulted in plasmid pJWpalTn (Fig. 1). Strain *P. putida* S12pal was constructed by introducing this plasmid in *P. putida* S12.

As a negative control, strain *P. putida* S12Tn1 was constructed. This strain contained the "empty" expression vector pTn-1.

The heterologous expression of yeast enzymes in bacteria is often impeded due to differences in, e.g. codon usage and post-translational modifications. In addition, PAL consists of four subunits that need to fold and interact properly for full activity (Calabrese et al. 2004). To investigate if active PAL was formed in *P. putida* S12, its functional expression was studied. Enzyme activity assays were performed with cell free extracts from induced and non-induced *P. putida* S12pal. It was shown that PAL is functionally expressed in *P. putida* S12pal, with a specific activity of 39±2 μmol min^{-1} g extract $protein^{-1}$ (results not shown). To our surprise, an uninduced culture of S12pal showed a PAL activity that was only 15% less than the PAL activity found in induced S12pal. The activities found are within the range of PAL activities found in *Escherichia coli* strains expressing PAL (Faulkner et al. 1994; Ørum and Rasmussen 1992).

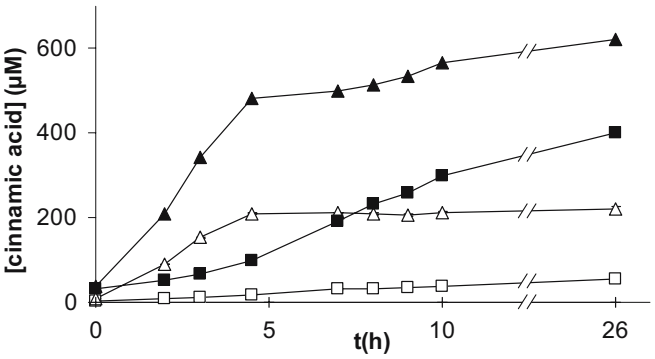

Fig. 2 Production of cinnamic acid by *P. putida* S12pal (*open triangle*, *open square*) and *P. putida* S12palM12 (*filled triangle*, *filled square*) grown in shakeflasks in MMG with (*open triangle*, *filled triangle*) or without supplementation with 1 mM phenylalanine (*open square*, *filled square*). The cells were grown (in triplicate) in 250-ml Erlenmeyer flasks containing 50 ml of medium at 30°C on a rotary shaking platform. Salicylate (0.1 mM) was added as an inducer. Extracellular cinnamic acid concentrations in the supernatant were determined by HPLC. *Error bars* represent ±standard deviation

Production of cinnamic acid from glucose by growing cells of *P. putida* S12pal

Since the functional expression of PAL in *P. putida* S12pal was established, the next step was to determine whether cinnamic acid could be produced from glucose by whole cells in different growth media. In *P. putida* S12pal the route of cinnamic acid production from glucose, except for the last conversion step, is via the phenylalanine biosynthesis pathway. Similar to *E. coli*, the biosynthesis of phenylalanine in *Pseudomonas* is tightly regulated (Gibson and Pittard 1968) by feedback inhibition.

In addition, exogenously supplied phenylalanine was rapidly degraded by *P. putida* S12 in MMG. A degradation rate of 4.5 μmol min^{-1} g cell dry $weight^{-1}$ was found (results not shown). From these results, we expected a low availability of phenylalanine in the cell for PAL which could form a major bottleneck in cinnamic acid production.

In order to determine the influence of phenylalanine availability on cinnamic acid production, the construct *P. putida* S12pal was grown in shakeflasks in MMG with or without 1 mM phenylalanine. In medium without phenylalanine, cinnamic acid did accumulate to a concentration of 71 μM (Fig. 2). However, the addition of phenylalanine to the medium caused a threefold increase of the cinnamic acid concentration to 226 μM. As expected, *P. putida* S12pal also produced 4-hydroxycinnamic acid. However, this product accumulated transiently and disappeared completely after 10 h of cultivation (results not shown). We found that *P. putida* S12 is able to use 4-hydroxycinnamic acid as a sole source of carbon (results not shown).

In conclusion, *P. putida* S12pal excreted significant amounts of cinnamic acid in the medium and the availability of phenylalanine in *P. putida* S12pal was shown to be the main bottleneck in cinnamic acid production.

Overexpression of DAHP synthase and the P-protein in *P. putida* S12

In order to increase phenylalanine availability, several genes of the phenylalanine biosynthesis pathway were overexpressed in *P. putida* S12. In *E. coli* overexpression of phenylalanine and/or tyrosine feedback insensitive variants of the genes *aroG* (DAHP synthase) and/or *pheA* (chorismate mutase/prephenate dehydratase, P-protein) in this pathway led to major increases of phenylalanine production (Frost and Draths 1995).

In *P. putida* the feedback-inhibition mechanism of DAHP synthase is not clear, and therefore this gene was cloned in an unmodified fashion. For the P-protein of *E. coli*, it is known that deletion of the C-terminal part results in feedback insensitivity (Zhang et al. 1998). Backman et al. (1990) showed that a tryptophan residue in the protein was crucial for feedback inhibition. The P-protein of *P. putida* KT2440 also contains a tryptophan residue (http://www.pedant.gsf.de; gi_26988500).

Consequently, in order to PCR amplify two DAHP synthases and a P-protein from genomic DNA of *P. putida* S12, we used primers based on the genome sequence of *P. putida* KT2440. Primers for the P-protein were designed in such a way that the sequence encoding the C-terminal part from the tryptophan residue was omitted.

Thus obtained genes were designated *dahp*1, *dahp*2 and *phe*A*, respectively. *Dahp*1 and *phe*A* were identical at the level of amino acids to DAHP synthase from *P. putida* KT2440 (gi_26989048) and the P-protein from *P. putida* KT2440 (gi_26988500). *Dahp*2 differed only in one amino acid from the other DAHP synthase from *P. putida* KT2440 (gi_26989799). Constructs were made containing *dahp*1, *dahp*2 and *phe*A* or combinations of these genes under control of the NagR/pNagAa promoter. Overexpression of these constructs in *P. putida* S12, however, did

not improve phenylalanine production (results not shown), nor cinnamic acid production.

Generation and high-throughput screening of *m*-fluoro-phenylalanine resistant *P. putida* S12pal mutants for enhanced cinnamic acid production

As overexpression of putative bottleneck genes did not increase the availability of phenylalanine in the cell for optimisation of production of cinnamic acid, an approach was followed by which a toxic phenylalanine analogue, *m*-fluoro-phenylalanine, was used to select for mutants that overproduce phenylalanine (Fiske et al. 1983). *m*-Fluoro-phenylalanine kills cells as it is incorporated into cell proteins, causing them to become dysfunctional (Pine 1978). This effect can be counteracted via overproduction of phenylalanine caused by, e.g. regulatory mutations. Such mutations include alteration of a feedback-sensitive enzyme in the aromatic amino acid biosynthesis route into a feedback-resistant variant (Fiske et al. 1983).

P. putida S12pal was subjected to random mutagenesis, by means of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) treatment, followed by selection on solid medium containing *m*-fluoro-phenylalanine. Thus a bank of 2×10^4 *m*-fluoro-phenylalanine resistant mutants was obtained. This bank was then screened for mutants with elevated cinnamic acid production by growing cells overnight in MMG with salicylate in wells of microtiterplates. The presence of cinnamic acid was determined by measuring the absorbance at 278 nm of the culture supernatants. The cinnamic acid production of 45 selected mutants was monitored in shake-flask experiments in which the cinnamic acid concentration was determined by HPLC. Mutant S12palM12 was found to accumulate the highest levels of cinnamic acid. The cinnamic acid production characteristics of this mutant were studied in further detail.

Production of cinnamic acid from glucose by growing cells of *P. putida* S12palM12

In order to compare *P. putida* S12palM12 with its parent strain S12pal, the production of cinnamic acid by *P. putida* S12palM12 in MMG was studied in shakeflasks. Also, the effect of phenylalanine supplementation was studied. *P. putida* S12palM12 accumulated 415 μM cinnamic acid in MMG (Fig. 2). Addition of phenylalanine to cultures of S12palM12 resulted in a 1.5-fold increase of the final cinnamic acid concentration to 637 μM. Thus, compared to the parent strain S12pal, the addition of phenylalanine had a far less dramatic effect on cinnamic acid production. These results indicate a higher intracellular availability of phenylalanine that, nevertheless, is still limiting the production of cinnamic acid.

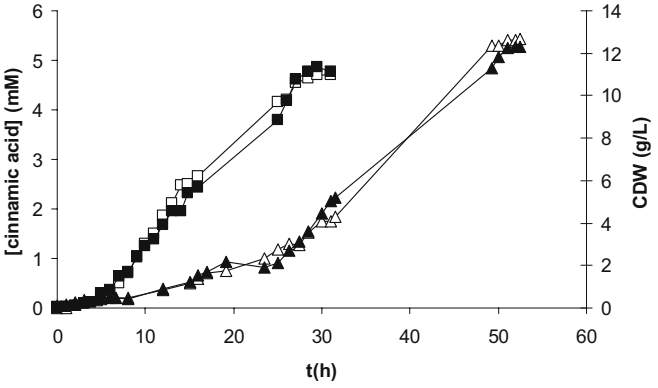

Fig. 3 Concentration cell dry weight (CDW; *filled square*, *filled triangle*) and production of cinnamic acid (*open triangle*, *open square*) by *P. putida* S12palM12 under nitrogen-limited conditions in fed-batch fermentations with glucose (*open square*, *filled square*) and glycerol (*open triangle*, *filled triangle*) as the carbon sources

Nitrogen-limited fed-batch cultivation

Subsequently, the production of cinnamic acid by *P. putida* S12palM12 was studied in fed-batch fermentations. Under nitrogen limitation with glucose as the sole carbon source, the culture yielded a final concentration of 5.0 mM of cinnamic acid (Fig. 3) with a $Y_{p/s}$ of 4.6% (Cmol cinnamic acid/cmol glucose).

As expected, under these nitrogen-limited conditions glucose (1 g l^{-1}) accumulated in the medium. However, we also found accumulation of up to 15 g l^{-1} of 2-ketogluconate (results not shown).

Increasing the feeding rate did not proportionally increase the growth rate and productivity, but led to even more accumulation of 2-ketogluconate.

The production of cinnamic acid was also studied in a nitrogen-limited fed-batch fermentation with glycerol as the carbon source. This culture yielded a final concentration of 5.4 mM of cinnamic acid (Fig. 3) with a $Y_{p/s}$=6.7% (Cmol cinnamic acid Cmol glycerol^{-1}). The maximum production rate ($q_{p,\,max}$) of this fed-batch fermentation was 1.0 μmol min^{-1} g cell dry weight^{-1}. With glycerol as the carbon source, *P. putida* S12 had a prolonged lag phase compared to the fed-batch fermentation with glucose as the carbon source (Fig. 3).

Discussion

The present study describes the production of cinnamic acid from glucose or glycerol by a variant of the solvent-tolerant bacterium *P. putida* S12 that expressed the *pal* gene from *R. toruloides*.

The main bottleneck in the bioproduction of cinnamic acid from glucose was the limited availability of intracellular phenylalanine. In *P. putida*, the availability of phenylalanine is the result of biosynthesis and degradation, and for an increase of the intracellular phenylalanine pool both aspects have to be dealt with.

In Pseudomonads the biosynthesis of phenylalanine shows similarities to that of *E. coli*, but it has been examined in far less detail. From studies with *P. aeruginosa* it has become clear that in the biosynthesis route of aromatic amino acids feedback control of key enzymes (by phenylalanine and tyrosine) is, like in *E. coli*, tightly regulated (Fiske et al. 1983; Ogino et al. 1982; Whitaker et al. 1982). In *P. aeruginosa* two key enzymes in this route, 3-deoxy-7-phosphoheptulonate (DAHP) synthase (E.C. 2.5.1.54) and prephenate dehydratase (E.C. 4.2.1.51), are under tight feedback control by phenylalanine and tyrosine. Prephenate dehydratase catalyses the formation of phenylpyruvate from prephenate. DAHP synthase is feedback inhibited by tyrosine (enzyme inhibition constant (K_i)=23 μM for both PEP and E4P) and to a lesser extent by phenylpyruvate (K_i=2.55 mM for PEP and 1.35 mM for E4P) (Fiske et al. 1983; Whitaker et al. 1982). Also, prephenate dehydratase and prephenate dehydrogenase are inhibited by phenylalanine and tyrosine, respectively (Dopheide et al. 1972; Gibson and Pittard 1968).

Overexpression of feedback insensitive *aroG* (DAHP synthase) and/or *pheA* (prephenate dehydratase) led to dramatic increases of phenylalanine production in *E. coli* (Frost and Draths 1995). In *E. coli* phenylalanine degradation is not an issue (Polen et al. 2005), thus allowing excretion of the compound.

In *P. putida* S12 overexpression of homologous (feedback insensitive) genes encoding DAHP synthase and the P-protein, which includes prephenate dehydratase activity, did not result in improved phenylalanine availability. This could be attributed to the fact that (1) phenylalanine biosynthesis in *P. putida* is more complicated than in *E. coli* (Byng et al. 1983; Fiske et al. 1983; Patel et al. 1977) and (2) the rapid degradation of phenylalanine in *P. putida* S12. Degradation of phenylalanine in *P. putida* occurs via the homogentisate pathway. Phenylalanine is converted into tyrosine, which is subsequently converted via 4-hydroxyphenylpyruvate into homogentisate. Homogentisate is further degraded to fumarate and acetoacetate (Arias-Barrau et al. 2004; Jimenez et al. 2002).

These results prompted us to choose the unprejudiced approach of random NTG mutagenesis. A selection on *m*-fluoro-phenylalanine medium was done in order to narrow down the number of mutants to be screened for optimised production of cinnamic acid. This approach had been successfully applied in *P. aeruginosa* to obtain mutants that accumulated more phenylalanine (Fiske et al. 1983).

Thus, we obtained a biocatalyst, *P. putida* S12palM12, with greatly improved cinnamic acid production characteristics after only one round of NTG mutagenesis (Table 2). In shakeflask experiments, both the yields of cinnamic acid in respect to glucose ($Y_{p/s}$) and to biomass ($Y_{p/x}$) were five to seven times higher compared to the parent S12pal. Also, the maximum specific cinnamic acid production rate ($q_{p,max}$) was eight times higher.

Fed-batch fermentation is often used in bioprocesses to improve growth efficiency, productivity and product yield by imposing a limitation (Lee et al. 1999). A nitrogen-limited fed-batch with glucose as the carbon source showed an increase in $Y_{p/s}$ of 145%, when compared to shakeflask experiments. The maximum production rate $q_{p,\ max}$ of this fed-batch fermentation was, as imposed by the chosen constant feeding rate, 1.7 μmol min^{-1} g cell dry $weight^{-1}$.

It is known that Pseudomonads extracellularly oxidise glucose to 2-ketogluconate via gluconate under high oxygen tension (Lessie 1984). 2-Ketogluconate is subsequently transported into the cell via a rate limiting step. This phenomenon impeded attempts to set up a glucose-limited fed-batch fermentation.

In order to develop an attractive 'green' production process for cinnamic acid, also from an economic point of view, product yields need to be further improved. In the near future, two aspects have to be dealt with: (1) cinnamic acid inhibits PAL activity (K_i=26 μM) and for this reason we expect that efficient removal of the product during production will optimise the productivity of the process; (2) improvement of phenylalanine availability in the biocatalyst. Although far less pronounced than in its parent, phenylalanine availability was still limiting cinnamic acid production in the optimised host. In this respect, it would be tempting to subject the biocatalyst to another round of mutagenesis. However, as in each round of mutagenesis adversary mutations may occur and further selection on fluoro-analogues of phenylalanine is not possible, we will engage in comparative proteomics and transcriptomics methodologies. By comparing the cellular response of several optimised mutants with the parental strain, we expect to obtain leads for further targeted improvement.

Table 2 Overview of the results obtained in shakeflask and batch experiments with *P. putida* S12pal and S12palM12

Medium	Method	Max [CA] (μM)		$Y_{p/s}$ (cmol%)		$Y_{p/x}$ (g g^{-1})		$q_{p,\ max}$ (μmol min^{-1} g CDW^{-1})	
		pal	M12	pal	M12	pal	M12	pal	M12
MMG	Flask	72	415	0.65	3.2	0.01	0.07	0.3	2.3
MMGp	Flask	227	627	n.a.	n.a.	0.05	0.09	1.1	3.2
ZHDP (glucose)	N-limited Fed-batch	n.d.	5,000	n.d.	4.6	n.d.	0.07	n.d.	1.7
ZHDP (glycerol)	N-limited Fed-batch	n.d.	5,400	n.d.	6.7	n.d.	0.07	n.d.	1.0

$q_{p,max}$ is calculated by multiplying $Y_{p/x}$ (μmol g CDW^{-1}) by μ_{max} (h^{-1})
MMG Mineral glucose medium, *MMGp* MMG supplemented with 1 mM phenylalanine, *ZHDP* adapted mineral medium, *CA* cinnamic acid, $q_{p,max}$ maximum specific cinnamic acid production rate, $Y_{p/x}$ yield in gram product per gram cell dry weight, μ_{max} maximum growth rate, $Y_{p/s}$ yield in cmol cinnamic acid per cmol glucose or glycerol used × 100%, *n.a.* not applicable, *n.d.* not done

Cinnamic acid is one of many substituted aromatics with very interesting industrial perspectives. This work could serve as the basis for the "green" production of such aromatics.

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