

Optimization of the solvent-tolerant *Pseudomonas putida* S12 as host for the production of *p*-coumarate from glucose

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Received: 11 August 2006 / Revised: 5 October 2006 / Accepted: 9 October 2006 / Published online: 17 November 2006
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Abstract A *Pseudomonas putida* S12 strain was constructed that is able to convert glucose to *p*-coumarate via the central metabolite L-tyrosine. Efficient production was hampered by product degradation, limited cellular L-tyrosine availability, and formation of the by-product cinnamate via L-phenylalanine. The production host was optimized by inactivation of *fcs*, the gene encoding the first enzyme in the *p*-coumarate degradation pathway in *P. putida*, followed by construction of a phenylalanine-auxotrophic mutant. These steps resulted in a *P. putida* S12 strain that showed dramatically enhanced production characteristics with controlled L-phenylalanine feeding. During fed-batch cultivation, 10 mM (1.7 g l⁻¹) of *p*-coumarate was produced from glucose with a yield of 3.8 Cmol% and a molar ratio of *p*-coumarate to cinnamate of 85:1.

Introduction

There is a growing interest in developing biotechnological processes for the production of chemicals from renewable resources (Frost 1994; Frost and Draths 1995; Maury et al. 2005). The main envisaged advantages of such “green” processes are the reduction of the use of fossil fuels and less

waste (CO₂) production (Anastas and Kirchhoff 2002; Maury et al. 2005). Various laboratories are developing whole-cell bioprocesses for the production of substituted aromatics such as *p*-hydroxybenzoate (Barker and Frost 2001), phenol (Wierckx et al. 2005), cinnamate (Nijkamp et al. 2005), *p*-coumarate, and *p*-hydroxystyrene (Ben-Bassat and Lowe 2004; Gatenby et al. 2002; Qi et al. 2003; Tang et al. 2003). Hydroxylated aromatics are a very important class of chemicals in terms of their broad application (Barker and Frost 2001; Matsusaki et al. 2005; Miyahisa et al. 2005). As the market for aromatics is huge and their chemical synthesis is oftentimes cumbersome, the bio-based production processes of these chemicals from renewable resources would provide a green and economically feasible alternative. We employ the solvent-tolerant *Pseudomonas putida* S12 as the host for these processes (Nijkamp et al. 2005; Wierckx et al. 2005).

P. putida is a metabolically versatile bacterium that has considerable potential for biotechnological applications (Jiménez et al. 2002; Nelson et al. 2002; Neumann et al. 2005; Wackett 2003). Examples of such applications are the bioconversion of toluene into 3-methylcatechol (Hüsken et al. 2001; Robinson et al. 1992), *o*-cresol (Faizal et al. 2005), and *p*-hydroxybenzoate (Miller and Peretti 2002; Ramos-González 2003), and the bioproduction of glucose to phenol (Wierckx et al. 2005) and cinnamate (Nijkamp et al. 2005). Our *P. putida* strain is solvent tolerant and is able to actively extrude a variety of compounds by means of a solvent pump (Isken and de Bont 1996; Kieboom and de Bont 2001; Rojas et al. 2001), which could serve as a driver of biocatalytic conversions by exporting the product from the cell into the medium.

In this report, we address the bio-based production of *p*-coumarate, also referred to as *p*-hydroxycinnamate, from glucose. In plants, *p*-coumarate is the precursor of various

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phenylpropanoids such as lignins, flavonoids, and coumarins (Hahlbrock and Scheel 1989; Hanson and Havir 1978). It is a useful bio-monomer for biological and medical applications as degradable plastic, orthopedic matrix, tissue engineering, and drug delivery systems (Kaneko et al. 2004; Matsusaki et al. 2001, 2005). Due to its reactive hydroxyl group, *p*-coumarate has a broader potential for industrial applications compared to cinnamate, especially for the synthesis of polymers.

Ben-Bassat et al. (2005) stated that chemical production methods for *p*-coumarate are expensive and result in large amounts of by-products. Methods of isolation and purification of *p*-coumarate from plants are also known (Benkrief et al. 1998); however, these methods are time-consuming and laborious and a more facile method of production is desirable for the commercial, large-scale synthesis of this compound. Leading chemical industries are indeed investing in research on the microbiological production of *p*-coumarate given the recent issuing of several patents (Ben-Bassat and Lowe 2004; Ben-Bassat et al. 2005; Gatenby et al. 2002; Qi et al. 2003; Tang et al. 2001).

The enzyme phenylalanine ammonia lyase (PAL, E.C. 4.3.1.5) catalyzes the conversion of L-phenylalanine and L-tyrosine to cinnamate and *p*-coumarate, respectively (Hodgins 1971). The production of cinnamate from glucose was previously achieved, with a yield of 4.6 Cmol%, upon introduction of PAL activity in *P. putida* S12. It was shown that *p*-coumarate was also produced, albeit transiently and in minute quantities (Nijkamp et al. 2005), due to efficient degradation of the compound.

The aim of the present study was to develop an optimized process for the production of *p*-coumarate from glucose in *P. putida* S12. Three main issues were addressed via metabolic engineering approaches: increasing the limited availability of L-tyrosine, preventing *p*-coumarate degradation, and preventing the accumulation of cinnamate as a by-product.

Materials and methods

Strains, plasmids, and culture conditions The strains and plasmids used in this study are shown in Table 1. The media that were used were Luria-Bertani broth (LB) (Sambrook et al. 1982) and a phosphate-buffered mineral salts medium as described previously (Hartmans et al. 1989). In mineral salts media, 20 mM glucose was used as the sole source of carbon (MMG), unless stated otherwise. For cultivation of L-phenylalanine auxotrophs (Phe[−] strains,) 10 mg l^{−1} L-phenylalanine was added to the medium (MMGP). Antibiotics were added to the media where appropriate at the following concentrations: ampicillin (Ap), 100 µg ml^{−1}; gentamicin (Gm), 10 µg ml^{−1} (MMG)

and 25 µg ml^{−1} (LB); and tetracycline (Tc), 10 µg ml^{−1} (for *Escherichia coli*) and 30 µg ml^{−1} (for *P. putida*). Shake flask experiments were performed in 250-ml erlenmeyer flasks containing 50 ml of MMG in a horizontal shaking incubator at 30°C. Cultures were inoculated with cells from an overnight culture to a starting cell density of approximately 0.1 g l^{−1} cell dry weight (CDW).

Fed-batch experiments were performed in 2-l fermentors (New Brunswick Scientific) using a BioFlo110 controller. Initial batch fermentation was started from a 50-ml inoculum of an overnight culture grown in MMG + 100 mg l^{−1} L-phenylalanine. For the batch phase (stage I), an adapted mineral salts medium was used with the following composition (per liter): 36 g glucose, 4 g (NH₄)₂SO₄, 3.88 g K₂HPO₄, 1.63 g NaH₂PO₄, and 20 ml trace element solution. The trace element solution had the following composition (per liter): 10 g MgCl₂·6H₂O, 1 g EDTA, 0.2 g ZnSO₄·7H₂O, 0.1 g CaCl₂·2H₂O, 0.5 g FeSO₄·7H₂O, 0.02 g Na₂MoO₄·2H₂O, 0.02 g CuSO₄·5H₂O, 0.04 g CoCl₂·6H₂O, and 0.1 g MnCl₂·4H₂O. Growth was controlled by continuous feeding of L-phenylalanine. After depletion of the initially added glucose, the L-phenylalanine feed was stopped and continuous feeding of glucose was started. The stirring speed was set to 200 rpm and air was supplied at 1 l min^{−1}. Dissolved oxygen tension was kept at 15% air saturation by automatic adjustment of the stirring speed and mixing with pure oxygen. Medium samples (5 ml) were taken during the fermentation to determine CDW, glucose, ammonium, *p*-coumarate, and cinnamate concentrations. CO₂ and O₂ concentrations in the offgas were measured using an Innova 1313 Fermentation Monitor. The pH was maintained at 7.0 with 4 N KOH and 4 N HCl.

Analytical methods Optical densities were measured at 600 nm (OD₆₀₀) with a Biowave cell density meter (WPA, UK). CDW concentrations were calculated from OD₆₀₀ values using the formula CDW (g l^{−1}) = OD₆₀₀ × 0.465. Cinnamate and *p*-coumarate concentrations were analyzed by high-performance liquid chromatography (HPLC) (Agilent 1100 system) using a Zorbax 3.5-µm SB-C18 column (4.6 × 50 mm) with acetonitrile/NaH₂PO₄ buffer (50 mM, pH 2, 1% acetonitrile) (25:75) as an eluent. Glucose concentrations were analyzed by HPLC (Waters) using an Aminex HDP-87 N column with 0.01 M Na₂HPO₄ as an eluent. NH₄⁺ concentrations were determined by cation-exchange chromatography (Dionex) as described before (Nijkamp et al. 2005).

DNA techniques The suicide vector pJQ200SK (Quandt and Hynes 1993) was used to construct a gene replacement vector for the *fcs* gene. Primers JW1–JW4 (Table 2), based on the DNA sequence of *fcs* from *P. putida* KT2440 (Nelson et al. 2002), were used to amplify the first 825 bp

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference and/or source
Strains		
<i>P. putida</i> S12	Wild type, ATCC 700801	Hartmans et al. 1990
<i>P. putida</i> S12pal	<i>P. putida</i> S12 containing plasmid pTacpal	Nijkamp et al. 2005 ^a
<i>P. putida</i> S12 C1	Derived from <i>P. putida</i> S12pal by NTG mutagenesis and <i>m</i> -fluoro-DL-phenylalanine selection	This study
<i>P. putida</i> S12 C2	<i>fcs</i> knockout strain derived from <i>P. putida</i> S12 C1	This study
<i>P. putida</i> S12 C3	Phenylalanine bradytrophic strain derived from <i>P. putida</i> S12 C2	This study
<i>E. coli</i> DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Sambrook et al. 1982
Plasmids		
pTacpal	Ap ^r Gm ^r , expression vector containing the <i>pal</i> gene under control of the <i>tac</i> promoter	Nijkamp et al. 2005 ^a
pTO1	Tc ^r , used for amplification of the <i>tetA</i> gene	Kieboom and de Bont 2001
pJQ200SK	Suicide vector, P15A <i>orisacB</i> RP4 Gm ^r pBluescriptSK MCS	Quandt and Hynes 1993
pJQfcs::tet	pJQ200SK containing a <i>tetA</i> interrupted copy of the <i>fcs</i> gene	This study

Ap^r Ampicillin resistance, Gm^r gentamicin resistance, Tc^r tetracycline resistance

^a Plasmid pTacpal has been erroneously replaced by pJWpalTn in the study of Nijkamp et al. (2005). pTacpal differs from pJWpalTn in that the *NagR/PnagAa* region has been replaced by the *tac* promoter (de Boer et al. 1983).

(designated *fcs1*) and the last 870 bp (designated *fcs2*) of the *fcs* gene. The tetracycline resistance gene (*tetA*) from vector pTO1 (Kieboom and de Bont 2001) was amplified using primers JW5 and JW6 (Table 2). pJQ200SK was digested with *NotI* and *BamHI* and *fcs1* and *fcs2* were digested with *NotI/XbaI* and *BamHI/XbaI*, respectively. The three DNA fragments were then ligated to yield pJQfcs1/2. pJQfcs1/2 was linearized with *XbaI* and treated with bacterial alkaline phosphatase. *TetA* was digested with *XbaI* and subsequently ligated into the *XbaI*-linearized pJQfcs1/2 vector to yield pJQfcs::tet. This construct was electroporated into *P. putida* S12 C1 and cells were plated on LB agar containing tetracycline. Colonies that were Tc^r were selected and replacement of the *fcs* gene by the *tetA*-disrupted *fcs* copy was confirmed by polymerase chain reaction (PCR) analysis, the inability to grow on *p*-coumarate as the sole source of carbon, and the production of *p*-coumarate.

Results

Isolation and characterization of a p-coumarate-overproducing strain of P. putida S12 *P. putida* S12 is able to

produce cinnamate and minute amounts of *p*-coumarate from glucose via L-phenylalanine and L-tyrosine, respectively, upon introduction and expression of the *pal* gene coding for phenylalanine ammonia lyase (Hodgins 1971; Nijkamp et al. 2005). It was shown previously that cinnamate production in such S12pal strain was greatly enhanced after a combination of NTG treatment and selection on *m*-fluoro-DL-phenylalanine (MFP), a procedure which selects for mutants with an enhanced metabolic flux towards L-phenylalanine (Nijkamp et al. 2005). Given the common biosynthetic pathway of L-tyrosine and L-phenylalanine, it was anticipated that this procedure would also yield mutants with an increased carbon flux to L-tyrosine and concomitant *p*-coumarate production.

Therefore, a library of 18,000 MFP-resistant mutants of *P. putida* S12pal, expressing *pal* from plasmid pTacpal (Table 1), was screened for *p*-coumarate production. These mutants were cultivated for 8 h in minimal glucose medium (MMG) in microtiter plates. The presence of *p*-coumarate in the culture supernatants was determined by measuring the absorbance at 310 nm. Six positive mutants were selected and cultivated in MMG in shake flasks to confirm increased *p*-coumarate production by HPLC analysis. The

Table 2 Primers used to construct a gene replacement vector for the *fcs* gene

Primer	Sequence (3'→5') ^a	Characteristics
JW1	gcgcggccgcgcatgcaacctgtcgagccactggcg	Start of <i>fcs</i> , forward primer, <i>NotI</i>
JW2	gcgtctagactcgcgcagattcgcgaaggtctcgg	Nucleotides 800–825 bp in <i>fcs</i> , reverse primer, <i>XbaI</i>
JW3	gcgtctagactacgcgaggtgttcttgcgccgcatc	Nucleotides 901–927 bp in <i>fcs</i> , forward primer, <i>XbaI</i>
JW4	gcggggtcctcaaggccgcaccttgccgtgcaatgc	End of <i>fcs</i> , reverse primer, <i>BamHI</i>
JW5	gcgtctagactcaggtcgaggtggcccg	Leader sequence of the <i>tetA</i> gene in pTO1 (Kieboom and de Bont 2001), forward primer, <i>XbaI</i>
JW6	gcgtctagagaattctcatgtttgacagcttattc	End of <i>tetA</i> in pTO1 (Kieboom and de Bont 2001), reverse primer, <i>XbaI</i>

^a Restriction sites are underlined.

best performing mutant was designated *P. putida* S12 C1, which produced *p*-coumarate at a maximum concentration of 90 μM after 10 h of growth (Fig. 1; Table 3). This is a 14-fold increase in production compared to the parent strain *P. putida* S12pal (Fig. 1; Table 3). However, after 24 h, almost all *p*-coumarate had been degraded.

Construction and characterization of a feruloyl-CoA synthetase-deficient derivative of *P. putida* S12 C1 To completely prevent *p*-coumarate degradation in strain S12 C1, the gene feruloyl-CoA synthetase (*fcs*) encoding the first conversion in the *p*-coumarate catabolic pathway in *P. putida* (Jiménez et al. 2002) was inactivated. To this end, plasmid pJQ200SK (Quandt and Hynes 1993) was used as a delivery system for gene replacement by homologous recombination of the wild-type *fcs* allele by a *tetA*-cassette-disrupted copy. *P. putida* S12 C1 was electrotransformed with this construct and the resulting Tc^r clones were tested for the ability to grow on *p*-coumarate. Several clones unable to utilize *p*-coumarate were isolated. The successful replacement of *fcs* with the inactivated copy (*fcs::tet*) was confirmed by PCR analysis (not shown). One mutant, designated *P. putida* S12 C2, was selected. This transformant was found to stably accumulate 224 μM *p*-coumarate during shake flask cultivation in MMG (Fig. 1; Table 3). However, also 350 μM of cinnamate was formed, indicating that a considerable flux of carbon was directed towards L-phenylalanine in strain S12 C2.

Generation and screening of a library of L-phenylalanine-auxotrophic mutants of S12 C2 for enhanced *p*-coumarate and decreased cinnamate production A strategy was chosen to prevent de novo synthesis of L-phenylalanine in S12 C2 to prevent the formation of the by-product

cinnamate. S12 C2, which was cured from pTacpal, was treated with NTG to obtain a large population of randomly created mutants. The mutants were plated on MMG medium agar supplemented with 1 mg l^{-1} L-phenylalanine, which was assumed to be a growth-limiting concentration for phenylalanine-auxotrophic mutants. Approximately 15% of the colonies showed reduced growth. Three thousand mutants forming pinpoint colonies were tested for their ability to grow in MMG supplemented with 100 mg l^{-1} L-phenylalanine or with 100 mg l^{-1} L-tyrosine. Four mutants able to grow only in the medium supplemented with L-phenylalanine (Phe^- strains) were selected. Growth was occasionally observed in MMG without L-phenylalanine after prolonged incubation (>48 h). As this effect was not consistently found, physiological adaptation was not obvious. This effect could rather be explained by reverse mutations (adaptive mutations) that are promoted by the severe starvation stress (results not shown).

After reintroduction of plasmid pTacpal into the phenylalanine-auxotrophic strains, *p*-coumarate production in MMG supplemented with 10 mg l^{-1} L-phenylalanine (MMGP) was monitored (Fig. 1; Table 3). One strain, *P. putida* S12 C3, showed a dramatically improved *p*-coumarate production: 860 μM of *p*-coumarate was produced in MMGP during cultivation in shake flasks. This was a fourfold increase in maximum *p*-coumarate concentration as compared to *P. putida* S12 C2. Moreover, in this strain, the final cinnamate concentration was 70 μM , a fivefold decrease compared to S12 C1 and S12 C2.

Optimization of fed-batch conditions for *p*-coumarate production by *P. putida* S12 C3 The production of *p*-coumarate by *P. putida* S12 C3 with glucose as the sole source of carbon was studied in fed-batch fermentations. As

Fig. 1 Maximum accumulation of *p*-coumarate (white bars) and cinnamate (black bars) in shake flasks by *P. putida* S12pal, *P. putida* S12 C1, *P. putida* S12 C2, and *P. putida* S12 C3. Media used were MMG (S12pal, S12 C1, and S12 C2) and MMGP (S12 C3). The cells were grown (in triplicate) in 250-ml erlenmeyer flasks containing 50 ml of medium at 30°C on a rotary shaking platform. Error bars represent the standard deviation

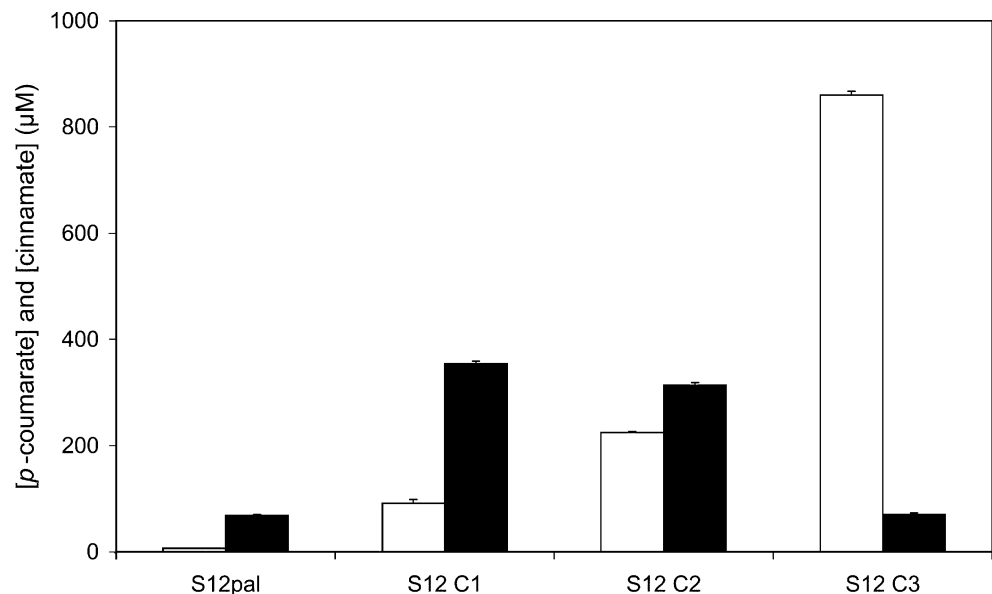


Table 3 Overview of the results obtained in shake-flask- and fed-batch-cultivated *p*-coumarate-producing *P. putida* S12 strains

Strain	[<i>p</i> -Coumarate] _{max} (μM)	[Cinnamate] _{max} (μM)	$Y_{p/s, p\text{-coumarate}}$ (Cmol%) ^a	$Y_{p/x, p\text{-coumarate}}$ (g g ⁻¹) ^b	$q_{p, \text{max}, p\text{-coumarate}}$ (μmol min ⁻¹ g ⁻¹ CDW) ^c
S12pal	7	72	0.05	8×10^{-4}	0.3
S12 C1	91	354	0.7	0.01	1.4
S12 C2	224	314	1.7	0.03	0.5
S12 C3	860	70	6.5	0.23	1.4
S12 C3 fed-batch	10,600	150	3.8	0.30	0.4

Shake flask experiments were performed in triplicate in 250-ml erlenmeyer flasks containing 50 ml of medium at 30°C on a rotary shaking platform. The maximum coefficient of variation was below 8%. Fed-batch data were obtained from a single representative experiment.

^a Yield in Cmol *p*-coumarate per Cmol of glucose used $\times 100\%$

^b Yield in gram *p*-coumarate per gram of cell dry weight

^c Maximum specific *p*-coumarate production rate calculated by the formula $q_p = r_p/M_x$ (Van't Riet and Tramper 1991), where r_p is the *p*-coumarate production rate (μmol l⁻¹ min⁻¹) and M_x is the biomass concentration (g l⁻¹)

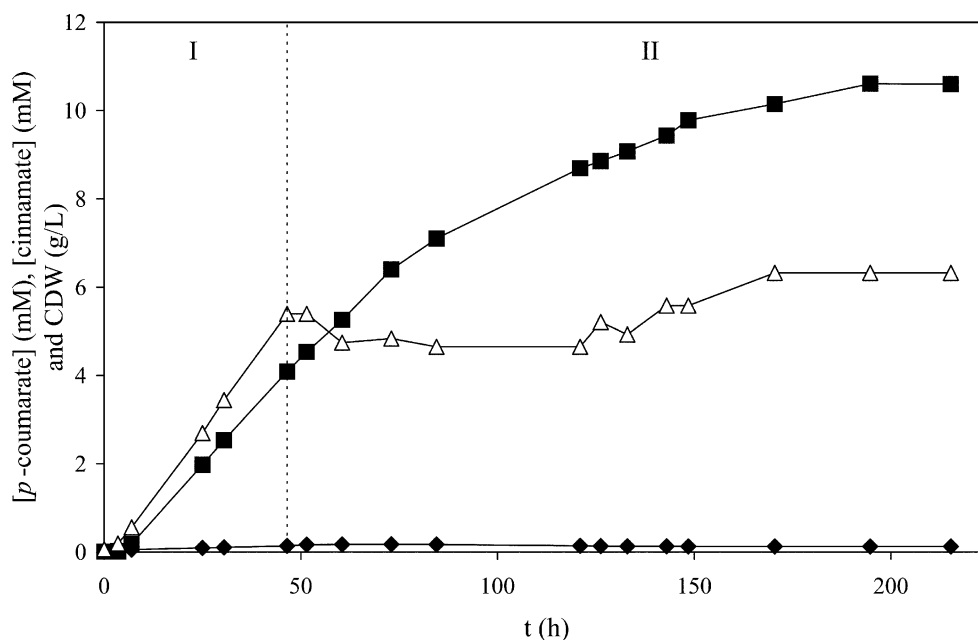
P. putida S12 C3 is Phe⁻, L-phenylalanine-limiting conditions were applied to control growth. Fed-batch experiments using different L-phenylalanine feed rates were performed to find the optimal balance between growth rate, biomass yield, *p*-coumarate yield, and prevention of cinnamate formation (data not shown).

Figure 2 shows the results of a typical fed-batch cultivation of S12 C3 under optimal conditions. During the first process stage (Fig. 2, I) L-phenylalanine was fed to the culture to allow for growth and *p*-coumarate production. An L-phenylalanine feed rate of 1.5 mg l⁻¹ h⁻¹ resulted in an optimal balance between the growth and production parameters. In the following stage (Fig. 2, II), upon depletion of glucose, the L-phenylalanine feed was stopped and a glucose feed was started at a rate of 1 g l⁻¹ h⁻¹.

During this stage, no formation of biomass was observed; however, production of *p*-coumarate proceeded at an almost constant rate of 87 μM h⁻¹. Both the *p*-coumarate and biomass yield on glucose reached their maximum at the end of stage II. After 120 h of fermentation, a slight increase in biomass concentration was observed. This may be attributed to reverse mutations, induced in the complete and prolonged absence of L-phenylalanine.

This fed-batch procedure resulted in a final concentration of 10.6 mM of *p*-coumarate (Table 3) with a maximum $Y_{p/s}$ of 3.8% (Cmol%). Only 150 μM of cinnamate was formed, resulting in a *p*-coumarate to cinnamate ratio of 85 mol mol⁻¹. The *p*-coumarate and biomass yield on L-phenylalanine were 30 mol mol⁻¹ and 75 g g⁻¹, respectively.

Fig. 2 Production of *p*-coumarate (closed squares), cinnamate (closed diamonds), and biomass (open triangles) in phenylalanine-limited fed-batch by *P. putida* S12 C3. Stage I: phenylalanine is fed to allow for biomass formation and *p*-coumarate production. Stage II: after depletion of initial glucose, phenylalanine feed is stopped and glucose is fed at a rate of 1 g l⁻¹ h⁻¹. Data were obtained from a single representative experiment



Discussion

In this study, a *p*-coumarate production process was developed by constructing a *P. putida* S12 strain that converts glucose via de-novo-synthesized L-tyrosine into *p*-coumarate by the enzyme phenylalanine ammonia lyase. L-Tyrosine availability, *p*-coumarate degradation, and by-product formation were critical issues that had to be addressed in optimizing this process.

In other *P. putida* strains, *p*-coumarate is converted via *p*-coumaroyl-CoA and *p*-hydroxybenzaldehyde to *p*-hydroxybenzoate by the enzymes of the *fcs/ech/vdh* gene cluster, feruloyl-CoA synthetase, enoyl-CoA hydratase, and vanillin dehydrogenase (Jiménez et al. 2002; Venturi et al. 1998). *p*-Hydroxybenzoate is subsequently converted by *p*-hydroxybenzoate hydroxylase and further degraded in the β -ketoadipate pathway to the TCA-cycle intermediates acetyl-CoA and succinyl-CoA (Jiménez et al. 2002; Overhage et al. 1999). The first conversions in the *p*-coumarate degradation pathway apparently exist also in *P. putida* S12. It was found that this strain rapidly degrades *p*-coumarate, and the *fcs* gene was identified in its genomic DNA.

P. putida S12 C1 grew poorly on *p*-coumarate as sole carbon source as compared to wild-type *P. putida* S12 (results not shown). Growth on *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate, intermediates in the degradation pathway of *p*-coumarate in *P. putida* (Jiménez et al. 2002; Overhage et al. 1999), as sole sources of carbon, was comparable to wild-type S12 (results not shown). These results indicated that accumulation of *p*-coumarate in S12 C1 had its origin in the hampered conversion of the compound in the first step(s) of the degradation pathway. In *P. putida* S12 C1, the *p*-feruloyl-CoA synthetase (*Fcs*) or enoyl-CoA hydratase (*Ech*) has presumably become dysfunctional due to the applied NTG mutagenesis procedure. Subsequent targeted disruption of the *fcs* gene of *P. putida* S12 resulted in a significant and sustained accumulation of *p*-coumarate, as demonstrated for mutant S12 C1, confirming its key role in the degradation of this compound.

Bio-based production processes for *p*-coumarate have been described for non-solvent-tolerant bacteria such as *E. coli* and *Pseudomonas aeruginosa* that expressed the *pal* gene from *Rhodospiridium toruloides* (Ben-Bassat and Lowe 2004; Gatenby et al. 2002; Qi et al. 2003; Tang et al. 2003). In these processes, cinnamate was also formed as a by-product due to dual specificity of *R. toruloides* phenylalanine ammonia lyase for both L-phenylalanine and L-tyrosine. Several approaches to address this issue have been suggested: (1) heterologous expression of *P. aeruginosa* phenylalanine hydroxylase that converts L-phenylalanine into L-tyrosine (Qi et al. 2003), (2) heterologous expression of cinnamate-4-hydroxylase that converts cinnamate to *p*-coumarate (Gatenby et al. 2002), and (3) modification of the *pal* gene such that the PAL substrate specificity for L-tyrosine is enhanced, resulting in a tyrosine ammonia lyase (Tang et al. 2003).

A fundamentally different approach was used in this study: instead of converting L-phenylalanine or cinnamate to L-tyrosine or *p*-coumarate, respectively, we constructed a bacterial host in which de novo synthesis of L-phenylalanine was eliminated. This approach has been shown to be profitable in the following respects: (1) cinnamate formation from phenylalanine is eliminated, (2) interruption of the carbon flux towards phenylalanine allows a greater portion of the carbon to be directed towards tyrosine and *p*-coumarate, and (3) growth and product formation can be controlled by applying specific L-phenylalanine feeding regimes.

‘Leaky’ or ‘reluctant’ L-phenylalanine auxotrophy was previously reported for *P. aeruginosa* (Patel et al. 1978). This was explained by the observation that, in this strain, dual biosynthetic pathways to L-phenylalanine and L-tyrosine exist (Patel et al. 1977, 1978). In *P. putida*, a similar organization of biosynthetic routes of aromatic amino acids exists (Byng et al. 1983), which explains the very low frequency of *P. putida* S12 Phe[−] mutants arising in our procedures. The Phe[−] phenotype of the constructed *p*-coumarate production host allowed for L-phenylalanine-controlled growth. This enabled us to optimize the balance between growth efficiency, productivity, and product yield during L-phenylalanine-limited fed-batch cultivation. Ben-Bassat et al. (2005) previously obtained a maximum of 1.3 g l^{−1} *p*-coumarate from glucose by *E. coli* in a fed-batch process. With *P. putida* S12 C3, we obtained a concentration of 10.6 mM of *p*-coumarate (1.7 g l^{−1}) and a biomass yield of 75 g g^{−1} under the most optimal conditions. As the cell densities in the process described by Ben-Bassat et al. (2005) were approximately tenfold higher, it can be derived that the yield on biomass ($Y_{p/x}$) is notably higher in our process. This implies that the conversion of glucose into *p*-coumarate is considerably more efficient than in the *E. coli*-based process.

During stage II of the fed-batch process, while the *p*-coumarate production rate remained constant, no formation of biomass was observed upon depletion of L-phenylalanine and constant glucose feeding. As no tyrosine accumulated at any stage of the process (not shown), product formation from previously accumulated tyrosine can be excluded. Thus, the *p*-coumarate production here can be classified as true de novo synthesis from glucose in non-growing cells. We found that the *p*-coumarate yield on glucose reached its maximum at the end of the zero-growth stage [$Y_{p/s, \max} = 3.8\%$ (Cmol/Cmol)], indicating that prolonging this phase would be one way to further enhance *p*-coumarate production. Based on maxi-

mum theoretical yield coefficients ($Y_{p/x}^{\max}$) calculated for phenylalanine biosynthesis from glucose (Baez-Viveros et al. 2004), it is expected that *p*-coumarate yield on glucose can maximally be improved by a factor of 10.

Investigations toward further process optimization that focus on reducing the competitive inhibition of PAL by *p*-coumarate [$K_i=18\text{ }\mu\text{M}$ (Hodgins 1971)] by applying in situ product recovery approaches are underway. Transcriptome analyses of different *p*-coumarate-producing S12 strains should also provide insights on possible bottlenecks in the metabolic machinery involved in the conversion of glucose into *p*-coumarate.

In conclusion, a bio-based process for the industrially important aromatic *p*-coumarate was greatly optimized in *P. putida* S12 through enhancing and re-routing the metabolic carbon flux from glucose towards this product via L-tyrosine. This work may serve as a basis for the optimized 'green' production of a variety of other substituted aromatics via this route.

Acknowledgements The authors thank H. J. Ruijsenaars for critically reading the manuscript.

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