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ORIGINAL PAPER

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Bioconversion of limonene to increased concentrations of perillic acid by *Pseudomonas putida* GS1 in a fed-batch reactor

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Abstract *Pseudomonas putida* GS1 is able to convert limonene to perillic acid (up to 64 mM,(11 g/l) when the bacteria is cultivated in fed-batch culture with non-limiting amounts of glycerol, ammonium, and limonene. *P. putid*a GS1 can use *p*-cymene as a single source of carbon and energy, and the enzymes that are responsible for the conversion of limonene to perillic acid belong to the degradation pathway of *p*-cymene. The *p*-cymene pathway of *P. putida* GS1 is very similar, if not identical, to the *cym* pathway of *P. putida* F1. The latter strain, and a recombinant *Escherichia coli* strain that carried the genes of the *cym* pathway of *P. putida* F1, also converted limonene to perillic acid. However, the final concentrations that were obtained in batch cultures with these two strains were lower than those obtained with *P. putida* GS1.

Introduction

Limonene is a cheap monoterpene that can be obtained in large amounts from peels of citrus fruit. Thus, it is a very suitable starting compound for the production of terpenoids with a higher economic value. Microorganisms have been isolated that transform limonene to terpenoids such as α-terpineol or carveol (for reviews, see Berger 1995; Krasnobajew 1984; van der Werf et al. 1997). Moreover, several microorganisms have been described that are able to use limonene as a single source of carbon and energy (Cadwallader et al. 1989; Chang and Oriel 1994; Dhavalikar et al. 1966; Rama Devi and Bhattacharyya 1977; van der Werf et al. 1999). Most of these strains initially oxidize the 7-methyl group of limonene to perillyl alcohol, which is subsequently converted to perillyl aldehyde and perillic acid by two dehydrogenases. This pathway was first characterized in *Pseudomonas putida* PL (Ballal et al. 1967, 1968; Dhavalikar et al. 1966). Besides this pathway, another metabolic pathway of limonene has been recently identified in *Rhodococcus erythropolis* DCL14. This strain degrades limonene via limonene-1,2-epoxide and limonene-1,2-diol (Barbirato et al. 1998; van der Werf et al. 1998, 1999).

The perilla intermediates of the limonene degradative pathway of *P. putida* PL may have chemotherapeutic activities against cancer (e.g., Crowell et al. 1991; Karlson et al. 1996; Schultz et al. 1997), and these products might therfore be of commercial interest. We previously described a solvent-resistant strain (*P. putida* GS1) that transformed limonene to perillic acid in amounts up to 18 mM (3.0 g/l) in the presence of a growth substrate such as glycerol (Speelmans et al. 1998). Perillic acid accumulated as a single, stable product with retention of configuration, which could be easily extracted from the culture medium. These properties make *P. putida* GS1 a very suitable biocatalyst for the production of perillic acid. Here, we show that the final concentration of perillic acid in the culture medium can be increased to 64 mM (11 g/l) by the use of a fed-batch bioreactor. Furthermore, we will show that the enzymes that cometabolically convert limonene are most likely derived from the metabolic pathway of *p*-cymene.

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Materials and methods

Strains, plasmids, and cultivation conditions

Pseudomonas putida GS1 (DSM 12264) converts limonene to perillic acid and was previously isolated from sludge (Speelmans et al. 1998). *P. putida* F1 grows with *p*-cymene and *p*-cumate, and the degradation route has been cloned and characterized (Eaton 1996, 1997). pRE847 contains a 10.22-kb *Hin*dIII fragment of *P. putida* F1 encoding the genes for conversion of *p*-cymene to *p*-cumate (Eaton 1997) and was a gift from R.W. Eaton (U.S. En-

vironmental Protection Agency, Fla.). *E. coli* JM109 (Yanisch-Perron et al. 1985) was used as a host strain for this construct.

P. putida strains were grown on Luria Bertani (LB) medium or on mineral E2 medium, as described by Lageveen et al. (1988), at 30 °C. *E. coli* JM109 was grown on LB or on M9 medium (Sambrook et al. 1989). Chloramphenicol (Cm) (30 µg/ml) and 1 mM of isopropyl-β-D-thiogalactoside (IPTG) were added to *E. coli* cultures carrying pRE847.

Fed-batch and batch cultivations were carried out with an ADI 3L bioreactor equipped with an ADI 1030 biocontroller (Applikon, Schiedam, The Netherlands) at 30 °C. The oxygen concentration in the culture was kept at 50% saturation by supplying filter-sterilized air when necessary. To prevent stripping, the airflow was saturated with limonene prior to addition to the culture by leading the airflow via a glass filter through pure limonene. The stirring speed was increased from 400–500 rpm at the beginning of the experiment to 1,000–1,100 rpm when the OD_{600} of the culture became higher than 2. The pH was adjusted to 7.0 with 1 M NaOH. When necessary, a few drops of Sigma antifoam 204 (Sigma-Aldrich, Zwijndrecht, The Netherlands) were added to the culture. During fed-batch cultivation, glycerol was added to the culture at a rate of 5.8 mM/h.

Preparation of cell extracts and enzyme assays

Crude cell extracts of *P. putida* were used to measure dehydrogenase activities with perillyl alcohol and perillyl aldehyde. For this, cells were harvested by centrifugation (15 min, 8,000 *g*, 4 °C) and washed with ice-cold 0.1 M Tris-HCl (pH 7.5) with 0.1 mM dithiothreitol. After resuspending the cells in a small volume of dithiothreitol, they were disrupted by sonification and centrifuged for 30 min in an Eppendorf centrifuge (13,000 *g*, 4 °C). The clear supernatant was used as a source of crude cell extract.

Alcohol and aldehyde dehydrogenase activities in crude extracts were determined spectrophotometrically by measuring the formation of NADH at 340 nm. The assay mixture for the alcohol dehydrogenase contained 80 mM glycine-NaOH, 1 mM perillyl alcohol, and 1.5 mM NAD at pH 11.0. The aldehyde dehydrogenase activity was determined in 0.1 M Tris-HCl with 10 mM cysteine, 2 mM perillyl aldehyde, and 1.5 mM NAD at pH 10.0. The pH of the assay mixtures was measured at the end of the assay and was unchanged.

DNA isolations and Southern hybridizations

Genomic DNA of *P. putida* was isolated according to Ausubel et al. (1994). DNA fragments of 1031 bp and 939 bp were amplified from genomic DNA of *P. putida* F1 with PCR by using primers against the *cymAb* gene (5′-ATGMGAAGTTTYTTYCA-3′ and 5′-GTRAAYTTRTCRTARAA-3′, M=A/C, R=G/A, Y=C/T) and the *cmtC* gene (5′-ATGGATATCACACTTCCC-3′ and 5′-TCATGCCAGTACCCCCTT-3′), respectively. The fragments were labeled with digoxigenin and used as probes in Southern hybridization experiment according to the manufacturer's instructions (Boehringer, Mannheim, Germany).

Analytical methods

Perillic acid and other terpenoids were extracted with chloroform from culture supernatant that was acidified to pH 1, after which they were analyzed by gas chromatography as previously described (Speelmans et al. 1998). The column temperature was set at 100 °C for 3 min, after which it was raised from 100 °C to 210 °C at 10 °C/min. Then, the temperature was further increased to 250 °C at 15 °C/min and kept at 250 °C for 8 min. The methylester of benzoate was used as an internal standard.

NH_4^+ was determined colorimetrically by using the Spectroquant kit (Merck, Darmstadt, Germany).

The concentration of glycerol was determined in the culture supernatant by reversed-phase high-performance liquid chromatography. The samples were acidified with 0.5 M H_2SO_4, centrifuged (5 min, 13,000 *g*), and filtered through a 0.2-µm cellulose acetate filter, after which they were injected onto an ion-300 column (Interaction Chromatography, San Jose, Calif.) and eluted with 3 mM H_2SO_4. Glycerol was detected with differential refractometry.

The protein content of cell extracts was determined with Coomassie brilliant blue with bovine serum albumin as a standard (Bradford 1976). The culture density was determined by measuring the optical density at 600 nm on a Ciba-Corning colorimeter 254, or by measuring the protein concentration. The latter was done with culture samples that were stored at –20 °C for several weeks. The samples were thawed and sonificated, after which the protein content of the disrupted cells was determined by using the Micro BCA protein assay reagent kit (Pierce, Rockford, Ill.).

Materials

To isolate (+)-perillic acid from cultures of *P. putida* GS1, the cultures were centrifuged (8,000 *g*, 15 min, 4 °C), after which the supernatant was acidified to pH 1 and extracted with ether. Perillic acid was removed from the ether layer by extraction with alkaline water (pH 10–14), precipitated by acidification, and isolated by filtration. The other terpenes and terpenoids that were used were obtained from sources that were previously described (Speelmans et al. 1998).

Results

Production of perillic acid in batch culture by *P. putida* GS1

It was previously shown that *P. putida* GS1 cometabolically converted limonene to perillic acid (Speelmans et al. 1998). A maximum concentration of 18 mM of perillic acid was obtained when *P. putida* GS1 was grown on E2 medium with 50 mM glycerol and 2.4% (150 mM) limonene. Higher concentrations of limonene were never obtained, although this concentration of perillic acid was not inhibitory for growth since the strain could still grow in spent medium that contained 18 mM of perillic acid when new carbon source (10 mM of succinate) was added (Speelmans et al. 1998). Since no limonene conversion was observed by cells that were growing in spent medium to which limonene and glycerol were added, it was suggested that feedback inhibition and/or repression determined the maximum amount of perillic acid that could be produced (Speelmans et al. 1998).

To test whether perillic acid also inhibited a further conversion of limonene in fresh medium, we grew *P. putida* GS1 on E2 medium with 0, 9, or 15 mM of perillic acid and 50 mM of glycerol in the presence and absence of limonene. The results confirmed that strain GS1 grows well in the presence of higher amounts of perillic acid (Fig. 1C, D). Besides, strain GS1 produced perillic acid in fresh medium both in the presence and absence of higher amounts of perillic acid. The initial rate of perillic acid production and the total amount of perillic acid that was produced were lower when perillic acid was already present at the beginning of the experiment (Fig. 1B).

Since production of perillic acid is still possible in the presence of 15 mM of this compound, other factors must

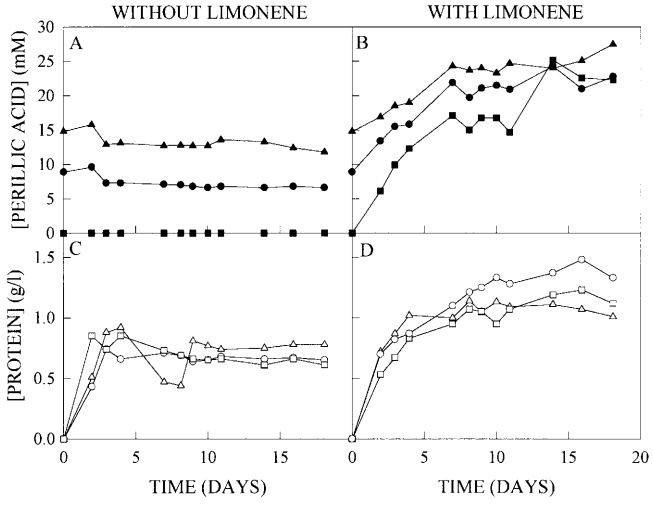

Fig. 1A–D Effect of perillic acid on growth and production of perillic acid in batch cultures of *P. putida* GS1. Cultures were grown on E2 medium with 50 mM of glycerol to which 0 or 2.4% of limonene, and 0, 9, or 15 mM of perillic acid was added at time 0. **A** and **B** show the concentrations of perillic acid that were measured in cultures that were grown in the absence and presence of limonene, respectively. ■ Concentration of perillic acid in cultures to which no perillic acid was added, ● concentration of perillic acid in cultures to which 9 mM of perillic acid was added, ▲ concentration of perillic acid in cultures to which 15 mM of perillic acid was added. **C** and **D** show the concentrations of protein that were determined as a measure of culture density in cultures that were grown in the absence and presence of limonene, respectively. □ concentration of protein in cultures to which no perillic acid was added, ○ concentration of protein in cultures to which 9 mM of perillic acid was added, Δ concentration of protein in cultures to which 15 mM of perillic acid was added

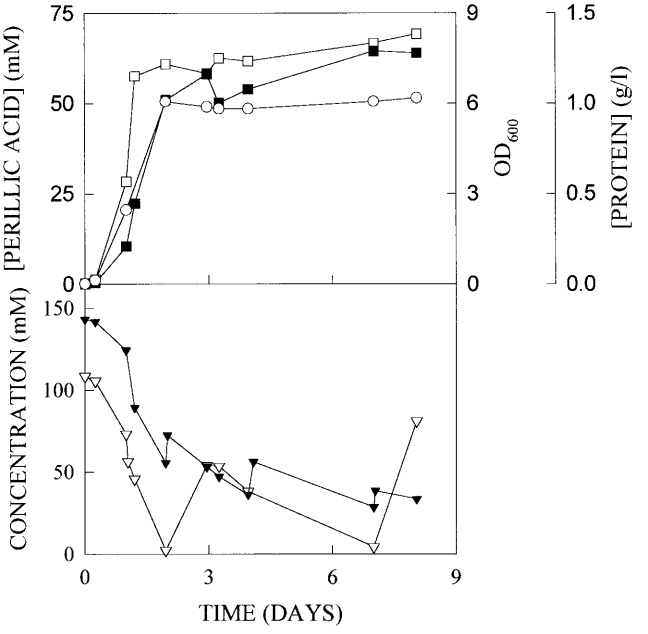

Fig. 2 Production of perillic acid by *P. putida* GS1 in a fed-batch bioreactor. The culture was inoculated in E2 medium with 100 mM of glycerol, 0.8% of limonene, and 75 mM of $(NH_4)_2SO_4$. Between day 1 and day 3, 284 mM of glycerol was added at a rate of 5.8 mM/h and extra limonene (0.8%) was added at t=2.0 and t=4.0. Besides, 133 mM of glycerol was added between day 7 and 8. To prevent limitations of other nutrients, trace elements and $MgSO_4$ (1×) were added at t=1.2 and t=7.0, and extra $(NH_4)_2SO_4$ was added at t=2 (17 mM), t=4.0 (20 mM), and t=7.0 (10 mM). The oxygen concentration was kept at 50% saturation by adding 3.75 l limonene-saturated air/min when needed. The stirring speed was increased from 500 rpm at t=0 to 1,000 rpm at t=1.0 and 1,100 rpm at t=2.0. The culture density is indicated as OD_{600} (□), and protein concentration (○), and the concentrations of perillic acid (■), glycerol (▽), and NH_4^+ (▼) are indicated

have determined cessation of the production of perillic acid. The pH in the batch cultures that contained limonene decreased from 7.0 to 5.9–6.2 depending on the amount of perillic acid that was produced (data not shown). This might have influenced the amount of perillic acid that could be produced. Furthermore, oxygen might have been limiting in the batch cultures, which were made in Erlenmeyer flasks. To exclude the influence of these factors, we repeated the experiment in a bioreactor in which the pH was adjusted to 7 and the oxygen concentration was kept at 50% saturation. In this culture, a final concentration of perillic acid of 18 mM was obtained, which was not significantly higher than the values obtained in Erlenmeyer flasks.

Production of limonene in a fed-batch culture by *P. putida* GS1

In all cases, the production of perillic acid slowed down when the culture reached the stationary phase, although limonene was still present in excess. Therefore, we repeated the experiment in the bioreactor under non-limiting growth conditions. For this, we grew the organism on non-limiting amounts of glycerol in the presence of 0.8% of limonene (Fig. 2). Within 2 days, the culture had reached an OD_{600} of 7.3, which corresponded to a biomass concentration of 1 g protein/l. The concentration of glycerol had decreased to 2.1 mM, and 51 mM of perillic acid had accumulated in the culture medium. This means that specific perillic acid production rate was 1.1 mmol perillic acid/g of protein per h during the first 2 days. The next day, the culture density hardly increased, although all necessary medium components were present in excess. The perillic acid concentration slowly increased to a final concentration of almost 64 mM (11 g/l).

The data suggest that 64 mM of perillic acid is inhibitory to growth of *P. putida* GS1. To test this, *P. putida* GS1 was grown on fresh E2 medium with 50 mM of glycerol in the absence and presence of about 65 mM of perillic acid and in the absence and presence of 0.8% of limonene (Fig. 3). Indeed, growth of strain GS1 was seriously hampered when 65 mM perillic acid was present. The strain still produced some perillic acid when 0.8% limonene was added to a culture that contained 65 mM perillic acid (Fig. 3).

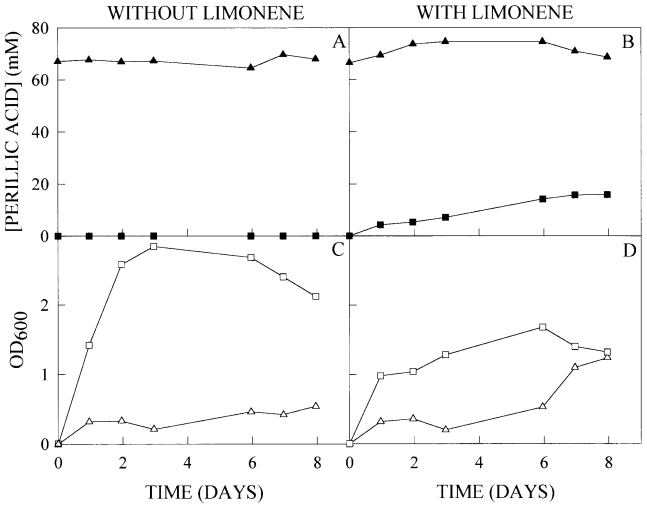

Fig. 3A–D Effect of 11 g perillic acid/l on growth and production of perillic acid in batch cultures of *P. putida* GS1. Cultures were grown on E2 medium with 50 mM of glycerol. **A** and **B** show the concentrations of perillic acid that were measured in cultures that were grown in the absence and presence of 0.8% of limonene, respectively. ■ Concentration of perillic acid in cultures to which no perillic acid was added at time 0, ▲ concentration of perillic acid in cultures to which 11 g perillic acid/l was added at time 0. **C** and **D** show the OD_{600} of cultures that were grown in the absence and presence of 0.8% of limonene, respectively. □ $OD_{600}$of cultures to which no perillic acid was added at time 0, Δ OD_{600} of cultures to which 11 g perillic acid/l of was added at time 0

Molecular characterization of limonene conversion by *P. putida* GS1

The conversion of limonene to perillic acid involves two dehydrogenase steps that oxidize the intermediates perillyl alcohol and perillyl aldehyde. The activities of these dehydrogenases could be measured in crude extracts made from batch cultures of *P. putida* GS1 that were grown for 3 days on E2 medium with 50 mM glycerol in the presence of 2.4% limonene. The activities were determined by measuring the reduction of NAD to NADH at 340 nm when perillyl alcohol or perillyl aldehyde was added (Table 1). When 2.4% *p*-cymene was added to a culture instead of limonene, the activities of the dehydrogenases were even higher, while no dehydrogenase activities could be detected in extracts of cells that were grown on glycerol in the absence of limonene or *p*-cymene (Table 1). The results suggest that both dehydrogenases are induced by the two monoterpenes. Since *P. putida* GS1 is able to use *p*-cymene as a single source of carbon and energy (Speelmans et al. 1998), it is likely that the enzymes that cometabolically convert limonene to perillic acid belong to the metabolic pathway of *p*-cymene.

P. putida F1 also grows with *p*-cymene, and the respective metabolic pathway has been characterized. Degradation of *p*-cymene starts with the oxidation of the methylgroup by a monooxygenase and two dehydrogenases, resulting in *p*-cumic acid (Eaton 1996, 1997). Analogous enzymes in *P. putida* GS1 are responsible for the conversion of limonene to perillic acid.

Table 1 Dehydrogenase activities with perillyl alcohol and perillyl aldehyde in cell-free extracts of *P. putida* GS1 grown on E2 medium with 50 mM glycerol in the absence or presence of 2.4% limonene or *p*-cymene

Monoterpene	Dehydrogenase activity with perillyl alcohol (U/mg of protein)	Dehydrogenase activity with perillyl aldehyde (U/mg of protein)
–	<0.02	<0.02
Limonene	0.47	0.14
p-Cymene	0.63	0.24

The genes of the *p*-cymene pathway of *P. putida* F1 have been cloned and sequenced (Eaton 1996, 1997). To determine whether the *p*-cymene pathway of strain F1 is similar to that of *P. putida* GS1, we amplified two fragments from the genome of strain F1 that contained most of the *cymAb* gene and the complete *cmtC* gene. These genes encode the reductase component of the *p*-cymene monooxygenase and the 2,3-dihydroxy-*p*-cumate 3,4-dioxygenase of the *p*-cymene pathway of *P. putida* F1, respectively.

The amplified fragments were used as probes in Southern hybridization experiments with *Eco*RI-, *Hin*dIII-, or *Pst*I-digested genomic DNA of *P. putida* strains GS1 and F1. Both probes hybridized with the genomes of both strains, and the sizes of the restriction fragments that hybridized with these probes were identical (results not shown). In addition, both sets of primers yielded PCR products with the proper size when the genome of *P. putida* GS1 was used as template, and the *cymAb* fragment of *P. putida* GS1 contained an *Eco*RI site at the same position as the fragment of *P. putida* F1 (results not shown).

The Southern hybridization and PCR experiments indicated that *p*-cymene is degraded by very homologous, if not identical, pathways in both *P. putida* strains. However, *P. putida* F1 grows with toluene as a single source of carbon and energy. The genes for the metabolism of toluene are encoded on the chromosome of *P. putida* F1 (e.g., Zylstra et al. 1988). Strain GS1 was unable to grow with this aromatic substrate, which means that the two strains are not identical.

Perillic acid production by strains that carry *cym* genes

Since *P. putida* F1 contains a similar or identical metabolic pathway for the degradation of *p*-cymene as *P. putida* GS1, and since this pathway is responsible for the conversion of limonene to perillic acid, it seemed likely that *P. putida* F1 is also able to convert limonene to perillic acid. Therefore, the capability of *P. putida* F1 to produce perillic acid was determined and compared with that of *P. putida* GS1. For this, both strains were grown on E2 medium with 50 mM glycerol and 2.4% limonene. *P. putida* F1 produced about 5 mM of perillic acid in 3 days. During the next 3 days, the concentration of perillic acid hardly increased but, after this period, an in-

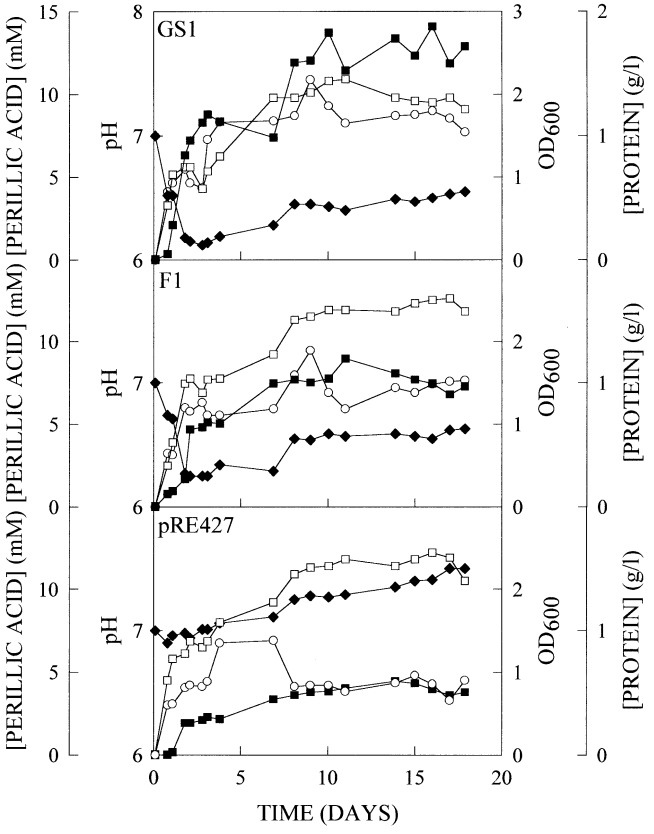

Fig. 4 Growth and production of perillic acid by *P. putida* strains GS1 and F1 and *E. coli* JM109(pRE847). Both *P. putida* strains were grown on mineral medium with 50 mM glycerol and 2.4% limonene. *E. coli* JM109(pRE847) was grown on LB medium with 2.4% limonene. ◆ pH, □ culture density measured as OD_{600}, ○, culture density measured as protein concentration, ■ concentration of perillic acid

crease of the perillic acid concentration to 9 mM was observed. With *P. putida* GS1 a similar pattern was observed, but the intermediate and final concentrations were higher (9 and 14 mM) (Fig. 4).

The ability to produce perillic acid was also determined for *E. coli* JM109(pRE847). This recombinant strain contains the genes of *P. putida* F1 for the conversion of *p*-cymene to *p*-cumic acid (Eaton 1997). *E. coli* JM109(pRE847) was grown on LB and M9 medium in the presence of different amounts of limonene. The highest amounts of perillic acid were produced when the strain was grown on LB medium with 0.24, 0.8, or 2.4% of limonene. The amounts of perillic acid were lower when lower amounts of limonene were added to the LB medium or when the strain was grown on M9 medium (Table 2). The experiment was repeated with LB medium and 2.4% limonene, and the formation of perillic acid by *E. coli* JM109(pRE847) was monitored (Fig. 4). The final concentration of perillic acid was lower than those obtained with the wild-type *P. putida* strains. These results indicate that *P. putida* GS1 is more suitable for perillic acid production than *P. putida* F1 or *E. coli* JM109(pRE847).

Table 2 Culture density and concentration of perillic acid in cultures of *E. coli* JM109(pRE847) grown on LB or M9 medium with varying amounts of limonene at 30 °C for 4 days

Limonene (%)	M9 medium		LB medium	
	OD_{600}	[Perillic acid] (mM)	OD_{600}	[Perillic acid] (mM)
0			1.50	0
0.016			1.50	0.7
0.08			1.18	3.7
0.24	0.46	0.9	0.97	7.3
0.8	1.18	4.5	1.07	7.9
2.4			1.21	6.7

Discussion

This report describes two different *P. putida* strains (F1 and GS1) that are able to convert limonene to high amounts of perillic acid. The terpenoid accumulates as a single, stable product in the culture medium. Neither of the two strains is able to grow with limonene as a single source of carbon and energy, which means that a growth substrate like glycerol has to be added to produce sufficient biomass for the conversion of limonene. Both strains were able to grow with the aromatic monoterpene *p*-cymene. Southern hybridizations and PCR analyses showed that both strains contain very similar, if not identical, pathways for the metabolism of *p*-cymene, which is oxidized to *p*-cumic acid by the monooxygenase and the two dehydrogenases of the *cym* pathway. *p*-Cumic acid is further metabolized to intermediates of the citric acid cycle by the enzymes of the *cmt* pathway (Eaton 1996, 1997).

The oxidation of limonene to perillic acid involves the same type of enzymatic steps as the oxidation of *p*-cymene to *p*-cumic acid. The dehydrogenases that oxidize perillyl alcohol and perillyl aldehyde are induced when *P. putida* GS1 is cultivated on glycerol in the presence of limonene or *p*-cymene. This indicates that the enzymes of the *cym* pathway are responsible for the oxidation of both terpenes to their corresponding acids. Moreover, a recombinant *E. coli* strain that carries the genes of the *cym* pathway converted limonene to perillic acid, which also shows that the enzymes that are responsible for the conversion of *p*-cymene to *p*-cumic acid also convert limonene to perillic acid.

The aromatic ring of *p*-cumate is oxidized to cumate dihydrodiol by a *p*-cumate 2,3-dioxygenase (Eaton 1996). Perillic acid is probably not a substrate for this dioxygenase since it accumulates in the culture medium. Microorganisms that metabolize limonene via perillic acid convert perillic acid via an ATP and coenzyme-A-dependent reaction, which eventually lead to 3-isopropenylpimelyl-CoA (Dhavalikar and Bhattacharyya 1966; Dhavalikar et al. 1966; Trudgill 1986). *P. putida* PL, which uses this pathway for growth with limonene, also grew with *p*-cymene (Dhavalikar and Bhattacharyya 1966). It was suggested that this strain uses the same en-

zymes for the oxidation of both limonene and *p*-cymene to their corresponding acids (Ballal et al. 1967, 1968), as we also observed with *P. putida* strains F1 and GS1. *Pseudomonas alcaligenes* IM88 (Teunissen and De Bont 1995) also grows with limonene and *p*-cymene. We recently isolated transposon mutants of this strain, according to Dennis and Zylstra (1998), that were no longer able to grow with limonene or *p*-cymene, but still grew with perillic acid. One of these mutants was further characterized. The transposon had disrupted a gene that was most homologous to the *p*-cumic aldehyde dehydrogenase gene of *P. putida* F1 (Eaton 1997), which means that this gene is necessary for growth with both monoterpenes. Besides, other transposon mutants were isolated that were unable to grow with limonene or perillic acid but still grew with *p*-cymene, suggesting that the transposon had disrupted a gene necessary for the metabolism of perillic acid. Since this mutation has no effect on growth with *p*-cymene, the metabolic pathways of *p*-cymene and limonene probably diverge after a certain point, most likely after the formation of the corresponding acids (Mars and Gorissen, unpublished results).

Of the three strains that were tested (*E. coli* JM109(pRE847) and *P. putida* strains F1 and GS1), *P. putida* GS1 accumulated the highest amount of perillic acid in batch cultures. The end concentration of 18 mM (3.0 g/l) of perillic acid that was obtained in batch cultures was not inhibitory for growth or production of perillic acid. However, production of perillic acid slowed down when the culture reached the stationary phase due to the limitation of a growth substrate. Speelmans et al. (1998) described that *P. putida* GS1 still grew in spent medium with this amount of perillic acid when a fresh amount of growth substrate was added, but no further conversion of limonene was observed. It was suggested that feedback inhibition and/or repression determined the maximum amount of perillic acid that could be produced (Speelmans et al. 1998). When grown in fresh mineral medium to which 18 mM of perillic acid was added, *P. putida* GS1 still converted limonene to perillic acid, which indicates that the inhibition of limonene conversion is not caused by the amount of perillic acid that is present. Indeed, when the period of non-limiting growth was extended in a fed-batch culture, the amount of perillic acid that accumulated increased to 64 mM (11 g/l). This amount of perillic acid strongly inhibited growth of *P. putida* GS1 when it was added to fresh medium, although some conversion of limonene to perillic acid still occurred.

Our present and previous results show that perillic acid can be produced as a single, stable product from the cheap starting material limonene. Concentrations as high as 11 g/l can be obtained in the culture medium by growing *P. putida* GS1 under non-limiting conditions, and a specific conversion rate of 0.18 g of perillic acid/g of protein per h can be obtained until the concentration of perillic acid is about 8 g/l. The organism converts limonene with retention of configuration, and perillic acid can be purified at low cost from an acidified culture supernatant by simple extractions (Speelmans et al. 1998). These properties make this method of biological perillic acid production of commercial interest.

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