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ORIGINAL PAPER

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Limonene bioconversion to high concentrations of a single and stable product, perillic acid, by a solvent-resistant *Pseudomonas putida* strain

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Abstract A newly isolated solvent-tolerant *Pseudomonas putida* strain converts (+)-limonene to high concentrations of a single and stable product, perillic acid. The presence of a cosubstrate is necessary for growth and perillic acid production. Glycerol appears to be the most suitable cosubstrate among those tested. An optimal combination of 150 mM limonene and 50 mM glycerol was found. Other factors that improve the extent and/or rate of bioconversion are the use of ammonia or urea as the nitrogen source, control of temperature at 30–34 °C and of pH at 7.0, as well as the use of emulsifiers to increase the bioavailability of limonene. Up to 18 mM (3.0 g · l^{-1}) perillic acid is produced, a concentration that is not growth inhibitory. The observations that a single product is formed in high concentrations and that it is not further metabolized make this limonene bioconversion of commercial interest.

Introduction

The monoterpene limonene [(4*R*)-(+)-4-isopropenyl-1-methylcyclohexene] is a cheap and readily available starting material for bioconversions to higher value compounds. It is the major constituent of citrus essential oils (Bowen 1975). The application of micro-organisms rather than use of chemical conversions offers the following advantages: the products formed are considered to be natural, the reaction can take place under mild conditions and multiple reactions can be performed in one step. Furthermore often a high degree of stereo- and regiospecificity is observed (Van der Werf et al. 1997; Krings and Berger 1998). Four major pathways of limonene bioconversion can be distinguished (Berger 1995): (1) oxidation of the methyl substituent to perilla compounds; (2) allylic oxidation to *cis*- and *trans*-carveols; (3) epoxidation of the double bond in the isopropenyl unit to α-terpineol; and (4) conversion of the ring double bond to the corresponding diol. As early as 1966 a soil pseudomonad was described, capable of converting limonene to several neutral and acidic products (Dhavalikar and Bhattacharyya 1966; Dhavalikar et al. 1966). Ever since that time many prokaryotic and eukaryotic micro-organisms have been described, which convert limonene to several products (for reviews, see Krasnobajew 1984; Trudgill 1986, 1994; Seitz 1994; Berger 1995; Van der Werf et al. 1997). Useful oxidation products include perillyl alcohol, perillaldehyde and perillic acid. These are naturally found in low quantities in citrus, lemon grass and perilla oils. They are utilized as flavourings (Fenaroli 1975) and as antimicrobial agents in foods and pharmaceuticals (Chadstein et al. 1992; Kim et al. 1995). Furthermore, a health claim is endowed on these terpenoids. When present in the diet of a rat, perillyl alcohol is observed to have a preventive role in the development of cancer (Crowell and Gould 1994; Crowell et al. 1994; Reddy et al. 1997; Stark et al. 1995).

The microbial toxicity and multiplicity of limonene metabolites results in low product concentrations and high downstream processing costs and this has impeded progress towards the bioproduction of monoterpene products from limonene (Van der Werf et al. 1997). Furthermore, subsequent degradation of the accumulated intermediates is often observed. A long fermentation time is needed before products are accumulated in larger quantities, which contrasts directly with the need for a short fermentation time because of the chemical instability and volatility of limonene (Van der Werf et al. 1997).

In the past decade bacterial strains resistant to solvents have been discovered and characterized (Inoue and

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Horikoshi 1989). These strains, usually identified as *Pseudomonas putida*, can adapt their membrane by conversion of *cis*- to *trans*-fatty acids in the presence of organic solvents such as toluene and styrene (Heipieper et al. 1992, 1994; Weber et al. 1994). Solvent-resistant strains therefore are excellent candidates for performing limonene bioconversions, since they are expected to be more resistant to the accumulated toxic terpenoids. Furthermore, this strain can be used in a multiphase bioreactor with limonene as a second phase (Witholt et al. 1990). In this paper the isolation of a new, solvent-resistant *P. putida* strain is described which converts (+)-limonene to large amounts (18 mM) of a single, stable product, perillic acid. The presence of a cosubstrate is needed and, by optimizing the culture conditions, the yield and rate of perillic acid production and efficiency of conversion that are obtained are among the most promising described so far.

Materials and methods

Isolation and identification of *P. putida* GS1

Micro-organisms were isolated from sludge by an enrichment technique using 5% (+)-limonene (v/v) (which corresponds to a theoretical concentration of about 300 mM) added directly to mineral E2 medium (Lageveen et al. 1988) as the sole carbon and energy source. This enrichment technique selects for solvent-tolerant and solvent-resistant Gram-negative bacteria. Bacteria were grown at 30 °C and at pH 7.0. After three transfers in liquid medium containing limonene, colonies were streaked to purity on Luria broth (LB) agar plates. Pure cultures were tested for their ability to accumulate products derived from the bioconversion of 50 mM (+)-limonene in liquid medium in the absence or presence of co-substrates. The strain which showed the greatest amount of accumulated products derived from (+)-limonene was identified by a BioLog, GN Microplate (Biolog, Hayward, USA) as a *P. putida* type A1 with a similarity of >0.95. This strain was subsequently named *P. putida* GS1 (DSM2 12264) and used in further experiments.

Cultivation conditions

LB (25 ml) was inoculated by a colony of *P. putida* GS1 and grown overnight at 30 °C, 250 rpm. Unless stated otherwise, this preculture was used to inoculate E2 medium containing 10 mM glycerol and 50 mM limonene and incubated at 30 °C and at 200 or 250 rpm. Although limonene is a rather insoluble substrate, present as a second phase (with an actual concentration of limonene not exceeding 50 μM), in this manuscript we express the amounts of limonene in the unit mole, to estimate conversion yields more easily. Growth was observed by following the changes in turbidity at 450 nm. An optical density at 450 nm (OD_{450}) of 1 was assumed to represent 0.175 $g \cdot l^{-1}$ biomass (Lageveen et al. 1988). Other hydrophobic substrates tested for cooxidation with glycerol were added at 50 mM, except toluene (0.05% v/v, about 5 mM) and perillyl alcohol, perillaldehyde, (+)-carvone, (−)-carveol, linalool, geraniol and α-terpineol (1 mM). In the experiments in which co-substrate dependency was determined, various carbon sources were added at a final concentration of 30 mM carbon. In the experiments in which nitrogen limitation or nitrogen source dependency was investigated, $NaNH_4HPO_4 \cdot 4H_2O$ was replaced by an equimolar amount of $Na_2HPO_4 \cdot 2H_2O$ and the nitrogen source was added separately. In the experiments in which the effect of pH was investigated, pH was adjusted after autoclaving with sterile NaOH or HCl. The pH was checked at the beginning and end of fermentation. In the experiments in which the effect of emulsifiers was determined, polyoxyethylene lauryl ether (Brij 35) or polyoxy ethylene sorbitan monolaurate (Tween 20) were added at final concentrations of 0.5 $g \cdot l^{-1}$. In the spent medium experiments, *P. putida* GS1 was grown in E2 medium containing the proper carbon and energy sources until late stationary phase or, when limonene was present, for 4 days. Cells were removed by centrifugation using sterile glass tubes (12,000 *g* for 20 min at 4 °C). The supernatant was cleared by filtration through 0.45-μm filters.

GC and GC MS analysis

Unless stated otherwise samples for GC and GC MS analysis were obtained in the following way: a sample of 2 ml was pipetted from below the surface (thereby excluding most of the limonene which is present as a second, upper phase) and acidified with 1 N HCl to a pH of 2–3. Chloroform (2 ml) was added and mixed by vortexing for 30 s. Separation of the phases was achieved by centrifugation (at room temperature for 5 min at 2000 *g*). The upper aqueous layer was removed. The chloroform layer was transferred to a glass vial and dried by water-free Na_2SO_4. The chloroform used for extraction contained as internal standard the methylester of benzoate, ranging from 0.025 to 0.05 $mg \cdot ml^{-1}$. Also sodium benzoate was used as an external standard (at a concentration range of 0.05 to 2 $g \cdot l^{-1}$), for calibration, and as a second internal standard, in which case it was added in known amounts to and coextracted with the samples.

Analysis of terpenes and terpenoids was performed using a Carlo Erba GC6000 gas chromatograph (Carlo Erba, Italy) equipped with a 25 m × 0.25 mm i.d. (d_f = 0.20 μm) Chrompack CP-58 CB column (Chrompack). Then 1-μl samples were injected by split injection (split ratio 1:40) at an injector temperature of 250 °C. The column temperature was raised from 50 °C to 250 °C at 10 °C/min, kept at 250 °C for 20 min, and finally raised to 275 °C at 10 °C/min. Detection was performed with a flame ionization detector (FID) at a temperature of 280 °C. GC/MS was performed using a Carlo Erba Mega GC linked to a QMD-1000 mass spectrometer by a direct interface. The GC column and temperature programmes used were the same as described above. Positive ion electron-impact mass spectra (70 eV) were obtained at a source temperature of 200 °C. The scan range was 25–300 Da with a scan rate of 2 s^{-1}. Chiral GC analysis of perillic acid formed upon bioconversion of (+)- and (−)-limonene and, as a reference, (−)-perillic acid was performed with a 25 m × 0.25 mm Hydrodex β-3P column (Macherey-Nagel, Düren, Germany). Samples of 1 μl were injected by split injection (split ratio 1:20) at an injector temperature of 230 °C. The column temperature was isothermic at 168 °C for 20 min, then raised to 220 °C at 2 °C/min, and kept at 220 °C for 10 min.

Materials

R-(+)-Limonene, myrcene, *S*-(+)-carvone, sodium benzoate, methylcyclohexane, toluene, Brij 35 and Tween 20 were obtained from Merck, Germany. Geraniol was obtained from Carl Roth, Germany. (*S*)-(−)-Limonene, (1*R*)-(+)-α-pinene, *p*-cymene, (−)-perillaldehyde, α-terpeniol, (−)-carveol, (*R*)-(+)-perillyl alcohol, octane and methylbenzoate were obtained from Aldrich USA. (±)-Linalool, (−)-perillic acid, and α-terpinene were obtained from Sigma, USA. (+)-Perillaldehyde was obtained from Fluka Switzerland. (+)-Camphor was obtained from ICN, USA.

Results

P. putida GS1 converts limonene to large amounts of perillic acid

P. putida GS1 was grown for 48 h in the presence of 10 mM glycerol and 50 mM (+)-limonene. In the

culture supernatant a product was detected, which was absent in control experiments in which (+)-limonene was incubated in the absence of cells, or in control experiments in which *P. putida* NCIMB 8248 [a solvent-resistant strain unable to grow on (+)-limonene as the sole carbon and energy source] was grown in the presence of glycerol and 50 mM (+)-limonene. This product was present in large quantities (1.23 g $\cdot$ L^{-1}). Identification by GC MS revealed that this product, which embodied 99% of product formed from (+)-limonene by *P. putida* GS1, was perillic acid. Trace amounts of myrcenic acid, derived from myrcene contaminating the (+)-limonene, and of neric acid, geranic acid and of 1-carbonyl-2-hydroxy-4-(1-methylethenyl)-cyclohexane were observed.

Accumulation of precursors of perillic acid, or of any further bacterial degradation products was not observed. These (more hydrophobic) precursors were also absent from the limonene phase. A customary route of perillic acid formation is via perillyl alcohol and perillaldehyde (Trudgill 1986; Fig. 1). If these compounds were added to the medium, *P. putida* GS1 was able to convert them entirely to perillic acid (data not shown), indicating that this pathway is also present in this organism.

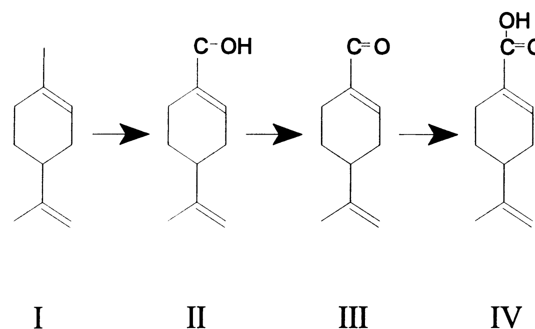


Fig. 1 Pathway of bioconversion of limonene to perillic acid. (*I* Limonene, *II* perillyl alcohol, *III* perillaldehyde, *IV* perillic acid.) (After Trudgill 1986)

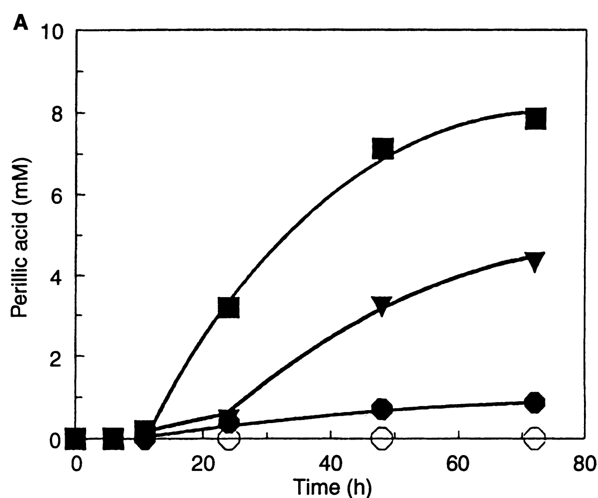
Substrate specificity of *P. putida* GS1 and optical purity of the products

Other substrates were tested for their conversion to acidic products by *P. putida* GS1 in the presence of 10 mM glycerol as cosubstrate after 48 h of growth. Myrcene was converted to myrcenic acid, and (−)-limonene was converted to perillic acid, albeit to lower final concentrations (3.6 and 1.2 mM, respectively). No accumulation of conversion products was detected with camphor, α-pinene, α-terpinene, *p*-cymene, octane, methylcyclohexane or toluene. Of these substrates only *p*-cymene was used as a carbon and energy source and was completely degraded. The other compounds were unaffected. Toluene (0.05%, v/v) was tolerated by this organism, so that it could be classified as solvent resistant (Weber et al. 1994). Of the terpenoids tested, (+)- and (−)-perillaldehyde and *R*(+)-perillyl alcohol were converted, whereas α-terpeniol, (+)-carvone, (−)-carveol, linalool, and geraniol were unaffected.

(+)-Limonene was converted into (+)-perillic acid and (−)-limonene was converted into (−)-perillic acid. In the latter case trace amounts of (+)-perillic acid were observed, probably derived from (+)-limonene contaminations in (−)-limonene.

Influence of cosubstrate and nitrogen source on the yield and production rate of perillic acid

P. putida GS1 barely grew on (+)-limonene as the sole carbon and energy source, except when present in high amounts (Fig. 2B). However, when this strain was grown in the presence of a cosubstrate, growth and perillic acid production were much improved (Fig. 2). In



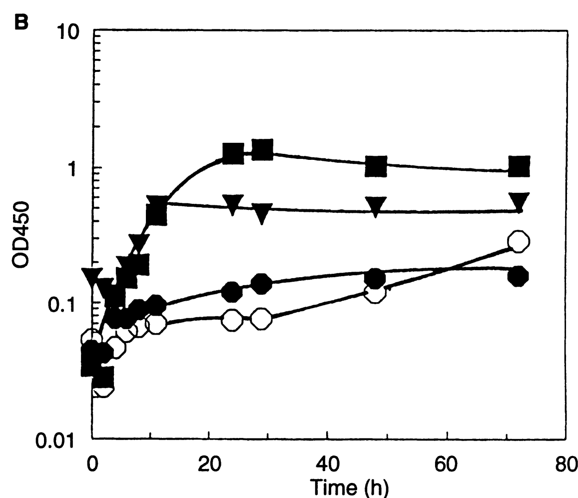


Fig. 2A, B Effect of glycerol on growth and perillic acid formation by *Pseudomonas putida* GS1. **A** Perillic acid formation in the presence of 50 mM limonene (●), 500 mM limonene (▼), 10 mM glycerol (○), and 50 mM limonene plus 10 mM glycerol (■). **B** Growth under the conditions described in **A** as measured by optical density at 450 nm

the presence of 10 mM glycerol as a cosubstrate the specific growth rate, μ, was increased from 0.032 to 0.127 h^{-1} and the maximal value of OD_{450} increased from 0.16 to 1.36 (Fig. 2B). Growth on glycerol in the absence of limonene was only observed after a long lag phase (> 48 h). The rate and amount of perillic acid formation in the presence of glycerol was increased from 0.018 to 0.198 mM · h^{-1}, and from 0.84 to 8 mM, respectively (Fig. 2A). The maximal amount of perillic acid was achieved after 120 h of fermentation. Subsequently the perillic acid produced remained stable in the culture for up to 3 weeks, which was the longest period tested.

Among the substrates tested glycerol was the most suitable cosubstrate for perillic acid production, yielding the highest rate of production (Fig. 3A). Expressed as production per hour and per gram biomass the rate of perillic acid production was comparable in the absence and presence of glycerol (0.37 g · g biomass^{-1} · h^{-1}). In the presence of succinate, lactate, acetate, glucose and butanol, a lower rate and amount of perillic acid production was observed as compared to glycerol, whereas an equal amount or more biomass was formed (Fig. 3). In LB hardly any perillic acid was formed (Fig. 3).

In addition, the nature of the nitrogen source influenced the rate and amount of perillic acid produced. Ammonia and urea were equally suitable nitrogen sources, whereas the presence of nitrate and glutamate resulted in a lower production rate (Fig. 4A). No effect on growth rate or growth yield was observed using these different nitrogen sources, except for an increase in μ in the presence of glutamate (Fig. 4B).

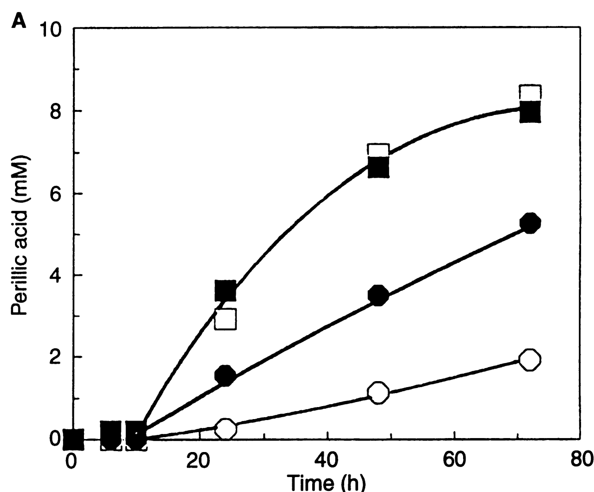
Fig. 3A, B Cosubstrate dependency of growth and perillic acid formation by *P. putida* GS1. **A** Perillic acid formation in the presence of 50 mM limonene and as cosubstrate glycerol (■), lactate (□), acetate (▽), succinate (○), butanol (◇), or glucose (▽), in the absence of a cosubstrate (●), or grown in LB plus 50 mM limonene (◆). Cosubstrates were present in concentrations corresponding to 30 mM carbon. **B** Growth under the conditions described in **A**

Optimization of (+)-limonene bioconversion by varying the ratio of cosubstrate to limonene

We determined the rate and amount of perillic acid produced with various concentrations of limonene (5, 15, 50, 150, and 500 mM) combined with various concentrations of glycerol (0, 10, 20, 50, and 100 mM). No nitrogen limitation was present under these circumstances. In Fig. 5 the maximal amount of perillic acid produced is shown. About 75–80% of this amount was formed in 72 h (data not shown). The greatest amount of perillic acid was produced in the presence of 150 mM limonene combined with 50 mM glycerol (18 mM). The production rate was increased to 0.39 mM · h^{-1}. A lower glycerol concentration decreased the final amount of perillic acid produced. Concentrations exceeding 50 mM glycerol did not result in any significant improvement. Concentrations of limonene exceeding 150 mM slightly decreased the amount of perillic acid formed, whereas growth was not impaired under these conditions. The maximal conversion of limonene was 30% and was observed in the presence of 50 mM limonene combined with 50 mM glycerol. The remainder of the limonene was either unaffected, evaporated (5–10%), or chemically oxidized (1–2%), depending on the incubation time. Limonene may also be incorporated into biomass but, if this happens, only in small quantities, since the remaining limonene could be quantitatively extracted from the culture. Surprisingly, the percentage of limonene bioconversion was not improved by lowering the concentration of limonene below 50 mM.

Influence of temperature and pH on product yield and production rate

The optimum temperature of growth for *P. putida* GS1 was typical for *P. putida* and was between 25 and 34 °C. The amount of perillic acid produced was greatest when grown at 25–30 °C (7.3 mM). Above 34 °C a sharp decline in perillic acid production was observed. If the rate



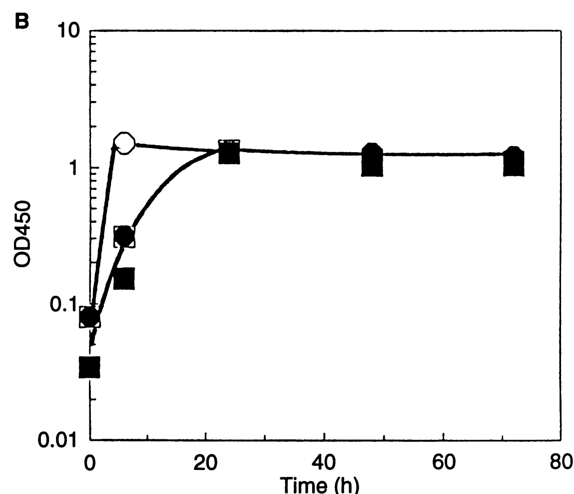


Fig. 4A, B Nitrogen source dependency of growth and perillic acid formation by *P. putida* GS1. **A** Perillic acid formation in the presence of 10 mM glycerol and 50 mM limonene in nitrogen-free E2 medium to which 17 mM NH_4Cl (■), or 17 mM $NaNO_3$ (●), 8.5 mM urea (□), or 8.5 mM glutamate without glycerol (○) was added. **B** Growth under the conditions described in **A**

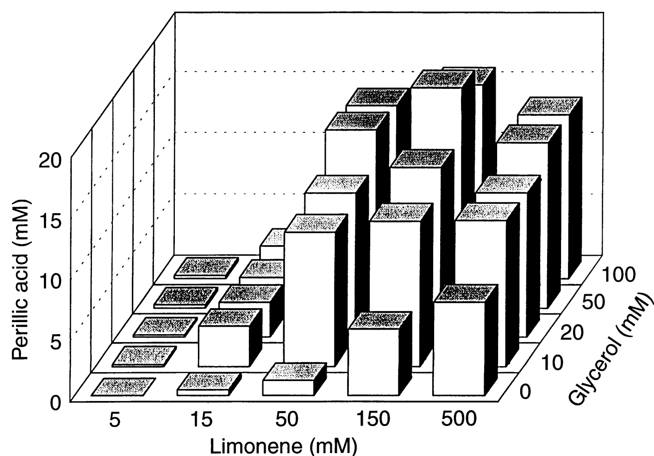


Fig. 5 Effect of combinations of different concentrations of glycerol and limonene on the formation of perillic acid by *P. putida* GS1 after 120 h

of production was considered, 30 °C was found to be optimal (rate of 0.21 mM · h^{-1}). However, again a sharp decline in production rate was observed above this temperature, necessitating strict temperature control (data not shown).

The optimum pH for perillic acid production was examined. In the range of from 6.15 to 8.3, μ and maximum value of OD_{450} were comparable. At pH values below 7.0 lysis was observed after this maximal OD value was reached. A sharp pH optimum of 7.0 was observed for the amount as well as for the rate of perillic acid production (7.7 mM, 0.19 mM · h^{-1}, respectively). Above pH 7.0 in particular, a rapid decline was observed, necessitating strict pH control (data not shown). Under normal growth conditions the pH of the strongly buffered E2 medium employed was not affected by the growth of and the amount of perillic acid formed by *P. putida* GS1.

Effect on lag phase of perillic acid formation of addition of emulsifiers and adaptation of the preculture

The production of perillic acid was delayed: a lag period of 10–24 h was observed before any perillic acid could be determined in the culture supernatant. During this period, glycerol enabled the cells to grow and form biomass. The highest rate of perillic acid formation under standard cultivation conditions was observed at the late exponential and early stationary growth phases. One of the reasons for the retarded formation of perillic acid may be the low availability of the water-insoluble, volatile limonene. It is conceivable that biosurfactants are required for emulsifying limonene, thereby increasing the bioavailability of limonene, and the biosynthesis for the biosurfactants may take some time. Therefore, the emulsifiers Brij 35, and Tween 20 were added to the medium. In the presence of emulsifiers the lag time for perillic acid formation was reduced from 12 to 6 h. Also the rate of production was increased 1.5-fold. The final amounts of perillic acid produced were comparable in the absence and in the presence of emulsifiers. The use of emulsifiers resulted in a reduction of the fermentation time needed to obtain a high concentration of perillic acid from 120 h to 48 h. At this time extra peaks in the GC chromatogram derived from emulsifiers had disappeared (data not shown) indicating that the emulsifiers had been consumed by the bacteria.

Pre-adaptation of cells is another way to shorten the lag phase. Cells were grown overnight on limonene plus glycerol and transferred to fresh medium containing these carbon and energy sources. A decrease in the lag phase of perillic acid formation of 12 to 6 h was observed. The decrease in the lag phase of growth was from 2 to 0 h. Concomitantly, the rate of production was increased 1.5-fold and the maximal amount of perillic acid formed (which was comparable) was accomplished more quickly, which reduced the fermentation time needed.

Limiting factors for perillic acid production

Although the bioconversion of limonene to perillic acid is already quite efficient, we wanted to determine the limiting factor for production in order to improve it. First we tested whether nutrient deprivation is a limiting factor. We therefore performed fed-batch studies in which extra amounts of limonene, glycerol, trace elements, inorganic phosphate, $MgSO_4$, nitrogen or combinations of these were added after 36, 48 or 72 h of growth of *P. putida* GS1 with 50 or 150 mM limonene and 20 or 50 mM glycerol. When 20 mM glycerol was used, extra addition of glycerol increased the amount of perillic acid produced; similarly when 50 mM limonene was used, extra addition of limonene increased production. These results are in agreement with those shown in Fig. 5. Other additions did not have any effect.

Another reason why not all limonene is converted to perillic acid could be that the membrane-permeable weak acid perillic acid has a growth-inhibiting effect, either by membrane disturbance and/or by ΔpH dissipation. Therefore experiments with spent medium containing various amounts of perillic acid were performed. *P. putida* GS1 was grown for 4 days in medium containing 50 mM glycerol, or 10 mM glycerol + 50 mM limonene, or 50 mM glycerol + 150 mM limonene. Cells were removed by centrifugation and filtration. The amount of perillic acid measured in the three spent media was 0, 7.8 and 18 mM, respectively. These three spent media and, as a control, fresh E2 medium were inoculated with a fresh preculture of *P. putida* GS1 and with 10 mM succinate as carbon and energy source, and growth was determined (Fig. 6). Growth of *P. putida* GS1 was comparable in fresh medium, in the 50 mM glycerol spent medium and in the spent medium containing 7.8 mM perillic acid. A lag period of 6 h instead of 2 h was observed in spent medium that contained 18 mM perillic acid. After this lag period, growth was resumed with the same μ and maximal OD values as with fresh medium (Fig. 6). At the end of the fermentation time, the concentration of perillic acid again was measured and found to be unaffected. In the absence of succinate growth was not observed (data not shown). These results indicate that, apart from the necessity of a period of adaptation to 18 mM, perillic acid does not inhibit the growth of *P. putida* GS1 and that perillic acid is not further degraded by the strain. Cells growing in spent medium containing 18 mM perillic acid to which glycerol (10 mM) and limonene (50 mM) was added did not further convert limonene into perillic acid.

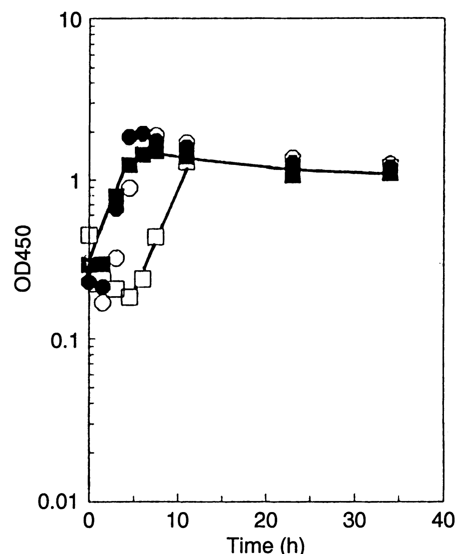


Fig. 6 Effect of perillic acid on growth of *P. putida* GS1 at 30 °C with 20 mM succinate as carbon and energy source. The medium was either fresh E2 medium (●) or spent E2 medium in which previously *P. putida* GS1 had grown in the presence of 50 mM glycerol (○), or spent E2 medium in which *P. putida* GS1 had grown in the presence of 10 mM glycerol and 50 mM limonene, containing 7.2 mM perillic acid (■), and on spent E2 medium in which *P. putida* GS1 had grown in the presence of 50 mM glycerol and 500 mM limonene, containing 18 mM perillic acid (□)

Discussion

This study describes the bioconversion of (+)-limonene to perillic acid by a newly isolated *P. putida* strain. This is one of the most promising bioconversions of limonene described so far when taking into account: (1) the efficiency of limonene conversion (30% of the added limonene is converted to perillic acid); (2) the rate of limonene bioconversion (48 h of fermentation is sufficient to obtain 80% of the maximal concentration when an overnight culture was inoculated 1/100); (3) the large amount of perillic acid produced (18 mM which corresponds to $3.0\ g \cdot l^{-1}$); (4) the fact that only one single product is formed (99% of the product formed is perillic acid); (5) the fact that perillic acid is chemically stable and not further degraded by the strain; and (6) the fact that the strain isolated appears to be solvent resistant. (This allows for much higher concentrations of substrate and products than with other bacteria and the use of this strain in a multiphase bioreactor.) A cheap downstream processing method can be envisaged. Besides chloroform, other cheaper and/or food-grade organic solvents such as hexane, pentane, petroleum ether and ethylacetate can also be used to extract perillic acid from an acidified aqueous sample. Subsequently perillic acid can be removed from the solvent by extraction with an alkaline aqueous solution (data not shown).

Bioconversion of limonene to 10.8 mM perillic acid is described for *P. gladioli*, now *Burkholderia gladioli* (Cadwaller et al. 1989). In addition α-terpineol and another acid are present in this case and after 4 days perillic acid is further metabolized by the strain. *P. putida* PL– (Dhavalikar and Bhattacharyya 1966; Dhavalikar et al. 1966) and *P. incognita* (linalool strain) (Rama Devi and Bhattacharyya 1977a, b) also produce many neutral and acidic products, including perillic acid, but the production kinetics of this compound are not known and further degradation of perillic acid is observed.

In order to convert limonene most efficiently to perillic acid *P. putida* GS1 needs a cosubstrate. Of the cosubstrates tested glycerol is the most suitable presumably because with this substrate no catabolite repression occurs. Organic acids such as succinate are strong catabolite repressors in *Pseudomonas* (MacGregor et al. 1992), and apparently also some repression occurs with butanol and glucose. An optimal ratio and concentration of glycerol versus limonene for perillic acid production was observed. Amounts of limonene greater than 500 mM did not enhance the amount of perillic acid formed; in addition, the percentage of bioconversion was not increased at limonene concentrations of less than 50 mM. A decrease in glycerol concentration decreased the final amount of perillic acid produced. Glycerol facilitates the production of biomass and so an increase in the concentration of enzymes involved in limonene conversion improves the rate of perillic acid production.

The concentration of perillic acid formed did not inhibit growth of *P. putida* GS1. Feedback inhibition and/or repression may play a role in determining the maximal concentration of perillic acid formed and so further improvements may be achieved by on-line removal of the product or in genetic engineering of the strain. Our next goal is to acquire a strain that is able to convert limonene to precursors of perillic acid, perillyl alcohol and perillaldehyde, since these terpenoids are also of commercial interest.

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