# INTEGRATED BIOPROCESS FOR THE PRODUCTION OF THE NATURAL ANTIMICROBIAL MONOTERPENE *R*-(+)-PERILLIC ACID WITH *P. PUTIDA*

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## Abstract

Experiments investigating the effects of perillic acid on bioconversion activity and growth of *P. putida* showed that product inhibition limits the production of perillic acid in fed-batch bioreactor to about 65 mM. To overcome this drawback, an *in-situ* product recovery bioprocess with anion exchange resin was developed. By using an integrated fed-batch bioprocess, which was set up by coupling a bioreactor with a by-pass *in-situ* recovery loop filled with anion exchange resin Amberlite IRA 410 Cl, perillic acid was extracted periodically from the broth and subsequently completely eluted from the resin with a mixture 60:40 (v/v) of ethanol and HCl 1M. This integrated bioprocess permitted to increase the final product concentration by 130 % to 150 mM after four days cultivation corresponding to a productivity of 37 mM/d.

## Introduction

Perillic acid, an almost odourless monoterpenoic acid, has a strong growth-inhibitory effect on bacteria and moulds. In addition to its broad antimicrobial spectrum, perillic acid shows antiallergenic properties making the acid a promising natural alternative to conventional preservatives, such as formaldehyde derivatives, for application in the cosmetics and pharma industries (1, 2). Limonene, the natural precursor of perillic acid, is a low priced by-product of the citrus processing industry and thus an environmentally friendly and cheap starting material for perillic acid synthesis. Since the regioselective oxyfunctionalisation of a C10-hydrocarbon such as limonene is not trivial to chemistry and usually demands harmful reagents such as heavy metal catalysts, biotechnology may be an interesting alternative. The microbial transformation of limonene frequently results in a large number of different metabolic products (3). However, the solvent tolerant gram-negative bacterium *P. putida* DSM 12264 is able to oxidise (+)-limonene to (+)-perillic acid via perillyl alcohol and perillaldehyde as metabolic intermediates and high product concentrations in the g/L range can be obtained under conventional cultivation conditions (4, 5). Nevertheless, the maximum product concentration, which has been so far achieved in a fed-batch biotransformation, is limited to about 65 mM after 9 days due to growth-inhibitory effects of perillic acid (5). This work reports on the development of an *in-situ* product recovery method based on anion exchange resins in order to further raise the productivity of the biocatalytic process by reducing the impact of product inhibition.

## Experimental

*Chemicals*. *S*-(−)-perillic acid (> 95 %), *R*-(+)-limonene (≥ 96 %), Amberlite IRA 410 Cl anion exchange resin, all reagents and solvents were purchased from Sigma-Aldrich, Germany.

*Microorganism and media.* Pseudomonas putida DSM 12264 (DSMZ, Braunschweig, Germany) was used. Terrific broth (TB) was used as complex medium for the growth inhibition assays and for the production of biomass. TB consisted of (in g/L): tryptone 12; yeast extract 24; glycerol 5; $KH_2PO_4$ 2.3; $K_2HPO_4$ 12.5; (pH 7). Phosphate buffer medium (0.1 M, pH 7) consisting of (in g/L) $KH_2PO_4$ 5.2 and $K_2HPO_4$ 10.7 and (in mM) glycerol 50 and limonene 150 was used for resting cells biotransformation assays. For all experiments investigating product inhibition effects, perillic acid concentration was adjusted in the media by adding respective aliquots of a highly concentrated alkaline perillic acid solution (403 mM, pH 10). E2 medium was used for limonene fed-batch biotransformations in the bioreactor, whose pre-cultures were prepared in LB medium (5).

*Product inhibition during the bacterial growth and the biotransformation.* To investigate the inhibitory effect of perillic acid on *P. putida* growth, cultivations were performed at different perillic acid concentrations between 0 and 119 mM in buffered TB medium to keep the pH at 7. The media (30 mL in 300 mL flasks) were inoculated with 5 % (v/v) of a pre-culture grown overnight in TB, and these cultures were incubated at 30°C, 240 rpm for 32 hours. For each perillic acid concentration, the maximum specific growth rate of the cultivation was determined by computer based differentiation of the growth curves using the software Origin (version 6.0, Microcal, USA) in order to estimate the perillic acid concentration above which cells do not grow by means of a linear mathematical model (6). To examine the action of perillic acid on *P. putida* limonene bioconversion, resting cell biotransformations were carried out with equal amounts of biocatalyst (1.1 g cdw/L; cdw: cell dry weight) but with increasing concentrations of perillic acid (0 mM to 30 mM). Biomass of *P. putida* was previously produced in 500 mL TB medium in 2 L Erlenmeyer flasks at 30°C, 240 rpm for 24 hours. The specific activity, which is expressed as Units (1U = 1 µmol perillic acid/min) per gram cdw, was determined by differentiation of the product formation kinetics (Origin, version 6.0, Microcal, USA).

*Fed-batch biotransformation.* The fed-batch biotransformation experiments with and without *in-situ* product recovery were carried out in 0.16 L culture volume with a FedBatchPro parallel fermentation system (DASGIP, Juelich, Germany), at 30°C. The dissolved oxygen concentration was kept above 60% saturation by adjustment of the stirring speed from 1000 rpm to maximum 2000 rpm. Air was supplied at a constant rate of 10 L/h to the reactor. The pH was maintained at 7.0 with 4 M NaOH. The culture was inoculated with 6% LB preculture ($OD_{600nm}$ = 7), previously prepared by overnight cultivation in 100 mL Erlenmeyer flasks at 30°C and 240 rpm, in 0.15 L E2 medium containing 100 mM of glycerol, 150 mM of ammonium and 4% (240 mM) of limonene. Glycerol was fed to the culture at an average rate of 16 mM/h between day 0.5 and day 6, and each 24 hours 75 mM pure limonene was added to the culture. To prevent limitations of other nutrients, 1 mL/L trace elements, 1 mL/L 1 M magnesium sulphate and 70 mM ammonium were daily added to the culture between day 2 and day 5.

*In-situ recovery of perillic acid in external loop and product purification. In-situ* recovery of perillic acid was performed by periodical recirculation of the culture at a flow rate of 3.6 L/h through a fluidized bed of anion exchange resin situated in an

external loop for 30 minutes per cycle when the perillic acid concentration approached 30 mM. A modified glass-chromatography column was used as fluidized bed column (15 mm inner diameter, length 220 mm, total volume 38 mL; purchased by Omnifit, Cambridge, England). The column was coupled between the bioreactor and the peristaltic pump (Ismatec, Glattburg, Switzerland) with Pharmed BPT tube (2.5 mm OD, 2.4 mm ID; Saint-Gobain, Vernet, France) and through the pump with Teflon tubing (4.3 mm OD, 2.9 mm ID; Hamilton, Bonaduz, Switzerland). The broth volume in the external recovery loop represented approximately 10% of the whole culture volume. The fluidized bed contained 20 g Amberlite IRA 410 Cl anion exchange resin. The bed of Amberlite IRA 410 Cl was aseptically changed with new resin (20 g) after the second and the third day from the start of the biotransformation. Each batch of resin loaded with perillic acid was successively eluted with 200 mL 1M HCl/ethanol (40:60 v/v) for 3.5 hours in a 500 mL shaking flask. To determine the additive perillic acid concentration during the integrated bioprocess, the quantification of the perillic acid content in the elution mixtures was performed by HPLC and was related to the reactor volume. This calculated concentration was added to the concentration of perillic acid found in the broth at the time when the recovery was performed. Afterwards, all elution mixtures containing perillic acid were collected and were filtered through a round filter paper (3 mm, diameter 15 cm; Whatman, England). The ethanol was evaporated in a rotary evaporator (55°C, 200 mbar) until appearance of white crystals in the aqueous 1M HCl solution. After vacuum filtration, the crystals of perillic acid were dried for 12 hours in a freeze dryer.

*Analysis of perillic acid.* For quantification of perillic acid, 1 mL of culture broth or 1 mL of elution agent was centrifuged at 14,500 g for 12 min and 10 µL supernatant was analyzed by HPLC (Shimadzu) comprising LC 10AT pump, M10A diode array detector (at 220 nm), and Lichrospher RP8 5µ 125 x 4 column (Phenomenex). The mobile phase was methanol / water 70:30 (v/v) containing 0.5 % 3M phosphoric acid, at 1 mL/min and 40 °C. Commercially available *S*-(−)-perillic acid was used as external standards.

## Results

*Inhibitory effects of perillic acid on P. putida.* Systematic investigations of the negative effect of increasing concentrations of perillic acid indicated that both growth and limonene biotransformation are inhibited by perillic acid. The maximum growth rate of non-transforming P. putida decreased linearly to complete inhibition at 165 mM perillic acid, while biotransformation of limonene with resting cells showed an exponential decrease of maximum specific activity from almost 8 U/g cdw without perillic acid to < 0.5 U/g cdw at > 25 mM perillic acid.

*Fed-batch biotransformation.* A fed-batch process was established to optimize the growth-associated production of perillic acid. A product concentration of 65 mM perillic acid was obtained with growing P. putida cells after 6 days (Figure 1) and with a maximum productivity of 27 mM/d during the first two days of cultivation. Product formation was not limited by glycerol, ammonium or limonene concentrations but by product inhibition at higher product concentrations.

*Ion exchange-based in-situ product recovery fed-batch bioprocess.* In order to avoid product inhibition during the limonene biotransformation and to enhance the production of perillic acid, an ion exchange-based *in-situ* product recovery fed-batch bioprocess was developed. Due to the dissociation behaviour of perillic acid (> 99 % dissociated form at pH 7) complete adsorption on anion exchange resins was

observed in preliminary experiments. The anion exchange resin did not influence cell integrity, composition of cultivation media or biotransformation pH (data not shown). By using an integrated fed-batch bioprocess, which was set up by coupling a bioreactor with a by-pass *in-situ* recovery loop containing a column filled with anion exchange resin Amberlite IRA 410 Cl (Figure 2), perillic acid was extracted periodically from the broth and subsequently completely eluted from the resin with a mixture 60:40 of ethanol and 1M HCl.

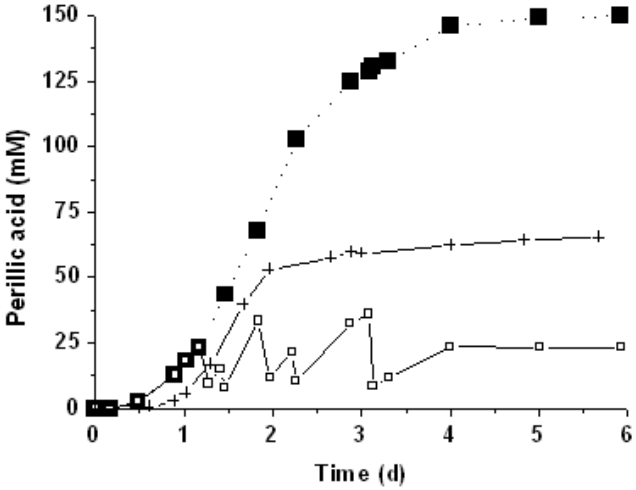


***Figure 1.*** *Comparison of perillic acid production kinetics with and without in-situ product removal. Perillic acid concentrations in the reactor during a conventional fed-batch biotransformation (+) and during an ISPR fed-batch biotransformation (□) as well as the resulting additive perillic acid concentration of the ISPR fed-batch biotransformation (■) are given.*

Coupling a limonene fed-batch biotransformation process with an ion exchange-based *in-situ* product removal permitted to get a final additive perillic acid concentration of 150 mM after four days cultivation corresponding to a maximum productivity of 37 mM/d. Figure 1 shows the perillic acid concentration in the broth and the additive perillic acid concentration for the integrated bioprocess.

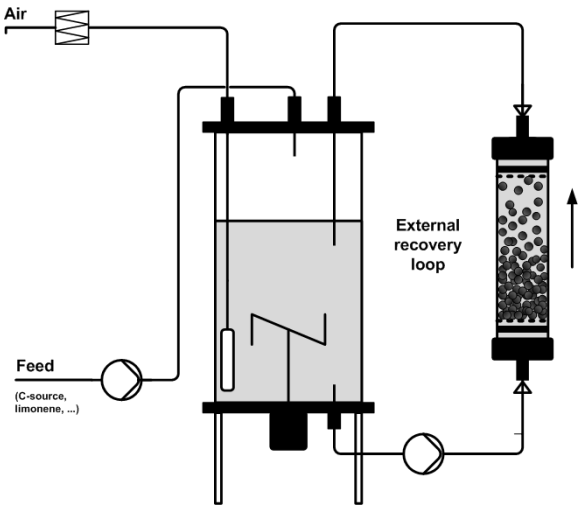


***Figure 2.*** *Scheme of the anion exchange-based in-situ product recovery fed-batch bioreactor for the production of perillic acid with P. putida DSM 12264.*

Downstream processing based on an ethanolic distillation delivered a product with a purity of ≥ 92 % and with only negligible loss of ≤ 3%. Using an integrated fed-batch bioprocess, it was possible to overcome product inhibition derived limitations of the limonene biotransformation. A periodical extraction of perillic acid by an external recovery loop with anion exchange resin led to a 2.3-fold increase in overall final perillic acid concentration and a 1.4-fold increase in productivity compared to the conventional fed-batch bioprocess previously described (4, 5). To the authors' knowledge, the results reported in this work correspond to the highest final product concentration and productivity achieved by microbial monoterpene oxyfunctionalisation up to now.

## References

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