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# Co-metabolic formation of substituted phenylacetic acids by styrene-degrading bacteria

Michel Oelschlägel *, Stefan R. Kaschabek, Juliane Zimmerling, Michael Schlömann, Dirk Tischler

*Interdisciplinary Ecological Center, Environmental Microbiology Group, TU Bergakademie Freiberg, Leipziger Str. 29, 09599 Freiberg, Germany*

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ABSTRACT

Some soil bacteria are able to metabolize styrene via initial side-chain oxygenation. This catabolic route is of potential biotechnological relevance due to the occurrence of phenylacetic acid as a central metabolite.

Thestyrene-degrading strains *Rhodococcus opacus* 1CP, *Pseudomonas fluorescens* ST, and the novel isolates *Sphingopyxis* sp. Kp5.2 and *Gordonia* sp. CWB2 were investigated with respect to their applicability to co-metabolically produce substituted phenylacetic acids. Isolates were found to differ significantly in substrate tolerance and biotransformation yields. Especially, *P. fluorescens* ST was identified as a promising candidate for the production of several phenylacetic acids. The biotransformation of 4-chlorostyrene with cells of strain ST was shown to be stable over a period of more than 200 days and yielded about 38 $mmol_{product}\ g_{celldryweight}^{-1}$ after nearly 350 days. Moreover, 4-chloro-α-methylstyrene was predominantly converted to the (*S*)-enantiomer of the acid with 40% enantiomeric excess.



## 1. Introduction

Phenylacetic acids represent an important class of compounds for several industries. They occur as natural ingredients in plants and fruits and are used as flavors and fragrances in cosmetics or food [14]. Additionally, these compounds have a high relevance for the pharmaceutical sector as precursors or drugs [29,51]. For example, α-methylated phenylacetic acids are currently applied as starting materials for the production of virostatic agents [49] or for receptor agonists and antagonists, e.g. for the histamine $H_2$ receptor [15,19]. Phenylacetic acids also serve as precursors for analgesics like diclofenac [40,41,44] or show already an analgesic effect like 4-isobutyl-α-methylphenylacetic acid which is also known as ibuprofen [8,12]. Furthermore, antibiotics on the basis of penicillin can be obtained from 4-hydroxyphenylacetic acid or non-substituted phenylacetic acid [9,11].

Because of the versatile applications of phenylacetic acids, different chemical strategies for their synthesis have been developed. The hydrolysis of phenylacetonitrile and its analogs in the presence of mineral acids at temperatures of up to 250 °C is one important way to produce phenylacetic acids [24]. Another important alternative is the carbonylation of benzyl chlorides in the presence of ruthenium(III) EDTA complexes [45], nickel catalysts [6], or rhodium-based catalysts [17]. Other chemical syntheses for compounds mentioned use α-hydroxynitriles [2], styrene and derivatives [8], or mandelic acid [29]. The α-methylated phenylacetic acids like ibuprofen are commonly obtained via corresponding phenylacetophenones from substituted- or non-substituted benzenes after initial Friedel–Crafts acylation [3,12,28,43].

As an alternative to chemical syntheses biotechnological strategies have been investigated to obtain aromatic acids. Gilligan et al. [16] transformed racemic 2-phenylpropionitrile via an amide to (*S*)-2-phenylpropionic acid applying a nitrile hydratase (EC 4.2.1.84) and a stereoselective amidase (EC 3.5.1.4) from *Rhodococcus equi* TG328. A remarkable enantiomeric excess (ee) of about 99% was achieved. The amidase of *Agrobacterium tumefaciens* d3 is also able to convert racemic 2-phenylpropionamide into the corresponding acid with an ee of 95% [48]. Sosedov et al. [42] have reported the direct hydrolysis of arylacetonitriles to phenylacetic acids by a recombinant arylacetonitrilase (EC 3.5.5.1) from *Pseudomonas fluorescens* EBC191.

Another biotechnological route to phenylacetic acids seems feasible applying styrenes. These styrenes are partly available in large amounts from the polymer industry [23] and can be converted by soil bacteria harboring enzymes of the styrene-catabolic pathway of side-chain oxygenation [32,36]. During side-chain oxygenation, the substrate styrene is initially oxidized into styrene oxide by styrene monooxygenase (SMO, EC 1.14.14.11, encoded by *styA*/*styB*) and subsequently transformed into phenylacetaldehyde by styrene oxide isomerase (SOI, EC 5.3.99.7, encoded

* Correspondence author. Tel.: +49 3731 394015; fax: +49 3731 393012
*E-mail address:* michel.oelschlaegel@ioez.tu-freiberg.de (M. Oelschlägel).

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by *styC*) (see Fig. 1). In a last step of this upper pathway, the aldehyde is oxidized to phenylacetic acid by phenylacetaldehyde dehydrogenase (PAD, EC 1.2.1.39, *styD*). The formed acid represents a substrate of the phenylacetyl-CoA ligase, which is the initial enzyme of the lower degradation pathway [7,37,38,46]. A modification of this pathway by deletion or substitution of enzymes is possible as mentioned by Hartmans et al. [20] or Toda and Itoh [47] . Previous studies have elucidated enzymes involved in the side-chain oxygenation of styrene in, for example, representatives of the genera *Corynebacterium*,*Rhodococcus*, *Pseudomonas*, *Sphingopyxis*, or *Xanthobacter* [5,20,22,33–35,47].

This study investigates the applicability of the styrene-degrading strains *Rhodococcus opacus* 1CP, *P. fluorescens* ST, *Sphingopyxis* sp. Kp5.2, and *Gordonia* sp. CWB2 as whole-cell biocatalysts for the co-metabolic production of substituted phenylacetic acids from corresponding styrenes.

## 2. Material and methods

### 2.1. Chemicals

Standard chemicals, substituted and non-substituted styrenes, styrene oxides, phenylacetaldehydes, and phenylacetic acids were purchased from Sigma–Aldrich (Steinheim, Germany), Merck KGaK (Darmstadt, Germany), AppliChem GmbH (Darmstadt, Germany), VWR International GmbH (Darmstadt, Germany), Riedel-de Haën (Seelze, Germany), Fisher Scientific (Loughborough, UK), Bio-Rad Laboratories GmbH (München, Germany), or Carl Roth (Karlsruhe, Germany) in highest purity available. The enantiomers of 4-chloro-α-methylphenylacetic acid were produced by the workgroup of Prof. Dr. Isamu Shiina (Tokyo University of Science) as described earlier [39].

4-Isobutyl-α-methylstyrene was obtained by Wittig-reaction of 4-isobutylacetophenone and in-situ-generated methylenetriphenylphosphorane according a protocol of [21]. The reaction product was purified by vacuum distillation and flash chromatography (silica gel, hexane) to yield the styrene as a colorless liquid. The retarded liquid contained 99% 4-isobutyl-α-methylstyrene (1.77 g, 10.2 mmol, 25.5% total yield). Purity was determined by silica gel chromatography and GC analysis while correct product formation was controlled via H NMR spectroscopy.

### 2.2. Bacterial strains and culture conditions

*R. opacus* 1CP (VKM Ac-2638 [18]), *P. fluorescens* ST (DSM 6290 [4,5]) *Sphingopyxis* sp. Kp5.2 (DSM 28731 [35]), and *Gordonia* sp. CWB2 (DSM 46758 [35]) were cultivated on mineral medium plates [10] in the presence of 20 g $l^{-1}$ glucose or in presence of gaseous styrene [34,35] for preservation.

Fed-batch cultivation was initially performed in 500-ml baffled flasks containing 50 ml mineral medium with 0.05% (w/v) yeast extract at 30 °C under constant shaking (120 rpm). In total 0.5–0.75 mmol glucose were added to the precultures as 0.25-mmol aliquots every 2–5 days. Cell growth was determined by the optical density at 600 nm ($OD_{600}$) and the cell dry weight.

The precultures were used to inoculate 1-l baffled flasks containing 200 ml mineral medium with 0.05% (w/v) yeast extract. Cultures were incubated at 30 °C and 120 rpm. In total 3–4 mmol glucose were added as 1-mmol aliquots during the first 3–4 days. In order to induce enzymes relevant for biotransformation, in total about 80 μmol styrene were added in 18–26-μmol portions through an evaporation adapter for further 5.5–6.5 days. The biomass obtained was applied immediately for biotransformation experiments. For this, biomass from two cultures of each strain was pooled and 200–400 ml of cells were harvested by centrifugation (5000 × *g*, 30 min, 4 °C). The cell pellet was washed twice with 50 ml of 25 mM phosphate buffer (pH 7.0) and centrifuged. The pellet obtained was suspended in 240–260 ml of fresh 25 mM phosphate buffer (pH 7.0) and the resulting cell suspension was used to investigate the substrate tolerance.

For long-time transformation of suitable substrates and strains, 1-l baffled flasks with 200 ml mineral medium containing 0.05–0.1% (w/v) yeast extract were inoculated with biomass from precultures, which was cultivated as described above, or directly by cells grown on solid medium or from cryo-cultures. Cultures were incubated at 30 °C and 120 rpm. In total 3.0–4.0 mmol glucose were added as 1.0-mmol aliquots during 10–11 days. Biomass obtained was, if appropriate, harvested as described above, pellet washed with sterile water, and cells subsequently suspended in 200 ml of fresh mineral medium without yeast extract. Cultures were initially incubated over 3–6 days in 1-l baffled flasks in presence of styrene (in total about 26–70 μmol, 18–26-μmol aliquots added through an evaporation adapter) for cell adaptation. Cells obtained were applied to investigate a fed-batch biotransformation in order to produce selected phenylacetic acids.

### 2.3. Investigation of substrate tolerance

20 ml of resuspended cells of each strain were distributed to 500-ml baffled flasks. 25 μmol of one of the following substrates were subsequently provided by means of an evaporation adapter: styrene, 3-chlorostyrene, 4-chlorostyrene, 4-fluorostyrene, α-methylstyrene, 4-chloro-α-methylstyrene, 4-isobutyl-α-methylstyrene. For the latter compound, also the direct addition to the culture medium was investigated because of its significantly reduced volatility compared to the other styrenes mentioned. Batches were cultivated at 30 °C and 120 rpm for 12 h. To determine product formation, samples of 750 μl were taken from the batches, centrifuged at 16.000 × *g* for 4 min, and supernatant analyzed by reversed-phase HPLC. To consider poor solubility of some products, especially of 4-chloro-α-methylphenylacetic acid and 4-isobutyl-α-methylphenylacetic acid in the culture medium, samples of 200 μl were diluted with 800 μl methanol. Diluted samples were mixed, centrifuged at 16.000 × *g* for 30 s, and supernatants analyzed by reversed-phase HPLC, too. The product yields determined after 12 h were normalized by the cell dry weight applied ($\mu mol_{product}\, g_{celldryweight}^{-1}$).

### 2.4. Fed-batch biotransformation for the production of selected phenylacetic acids

200 ml of a styrene-induced cell suspension of *P. fluorescens* ST were incubated with in total 3630 μmol 4-chlorostyrene which

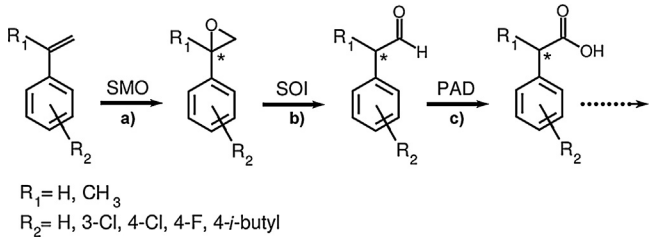


**Fig. 1.** Biotransformation of styrene and substituted analogs to phenylacetic acid(s). Styrene (R1, R2 = H) is transformed to phenylacetic acid through enzymes of side-chain oxygenation by the following steps: (a) initial epoxidation to styrene oxide by styrene monooxygenase (SMO), (b) isomerization to phenylacetaldehyde by styrene oxide isomerase (SOI), (c) oxidation by phenylacetaldehyde dehydrogenase (PAD) (reviewed by [32,36]). A star indicates the formation of a stereocenter in case of R1 = $CH_3$. The suitability of the catabolic route was tested for the formation of 3-chloro-, 4-chloro-, 4-fluoro-, α-methyl-, 4-chloro-α-methyl-, and 4-isobutyl-α-methylphenylacetic acid.