Microbial Formation of Substituted Styrenes

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Dedicated to Professor Klaus Kieslich on the occasion of his 60th birthday

Cinnamic Acid Derivatives, Biotransformation, Decarboxylation, Styrenes

Various mono- and disubstituted cinnamic acid derivatives and aromatic carboxylic acids with saturated side chains were incubated mainly with *Bacillus, Candida, Hansenula,* and *Saccharomyces* strains. The cinnamic acids carrying a hydroxy- and/or a methoxy group at the 3- and/or 4-position of the benzene ring were decarboxylated with high yields. Most of the reactions were terminated within 24 to 48 h. Substitution at other ring positions afforded also decarboxylation, but at much lower yields. Derivatives with other residues like methyl, chloride, or bromide were not transformed to the respective styrene. None of the saturated aromatic carboxylic acids could be decarboxylated by the strains used.

Introduction

Already in 1908 it was reported that cinnamic acid could be converted to styrene by *Penicillium glaucum* and *Aspergillus niger* [1]. Since then several reports appeared on the microbial or enzymatic decarboxylation of cinnamic acid derivatives [2–4, 11], which are widely distributed in higher plants [5]. The resulting styrenes occurred in a wide variety of alcoholic beverages [6], like wine and beer, resulting from fermentation of plant material with yeast [7]. Some styrenes are also constituents of the flavor of soya sauce [8], cloud- [9] and blueberries [10].

Our attention was focused on the specificity of some selected microorganisms and their inherent decarboxylases towards several cinnamic acid derivatives and corresponding aromatic acids with saturated side chains. In this paper we will report our investigations on the microbial non-oxidative decarboxylation by strains of *Candida, Hansenula, Bacillus,* and *Saccharomyces* and their substrate specificities.

Materials and Methods

Microorganisms kept in our department originated from the Deutsche Sammlung von Mikroorganismen (DSM) and from the American Type Culture Collection (ATCC). They were maintained on agar slants at 4 °C or frozen in liquid nitrogen. Medium was: 10 g glucose, 20 g malt extract, 10 g peptone, 3 g yeast extract in 1.0 l water, final pH 5.7. Transformation medium was composed of 5 g glucose, 2 g peptone, 5 g malt extract, 1 g yeast extract in 1.0 l water, final pH 5.7.

One loop of organisms from the slants were used to inoculate 20 ml of the same medium held in 100 ml Erlenmeyer flasks and the cultures were incubated for 48–72 h at 27 °C and 140 rpm. For analytical transformation reactions about 2 ml of the cultures were transferred to 20 ml transformation medium in 100 ml Erlenmeyer flasks. After incubation for 48 h under the same conditions the substrate solution was added.

The substrates were dissolved in dimethylformamide (DMF) at a concentration of 100 mg/ml and 100 µl aliquots were added to the cultures (10 mg substrate/20 ml medium). The reactions were followed by withdrawing 1 ml samples at various time intervalls. They were extracted with 200 µl ethyl acetate, 20 µl aliquots were applied to HPTLC and developed with dichloromethane/acetone (95/5, v/v). Products were detected by UV light and in addition the styrene derivatives gave deep blue spots after spraying the plates with vanillin-sulfuric acid and heating to 100 °C for 5 min.

Preparative scale experiments were carried out in 1-l Erlenmeyer flasks containing 200 ml of transformation medium. Inoculations were done by addition of a preculture (10% v/v), substrate concentrations were 0.5 mg/ml medium. During the course of fermentation the flasks were incubated at 27 °C and 140 rpm. A scaled-up fermentation of 15 g ferulic

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acid was performed using a stirred fermenter (B 20, B. Braun) with an actual working volume of 20 l medium (100 rpm, 0.1 vvm air).

Chemicals were obtained from Aldrich and Janssen and are of highest purity available.

Culture medium and mycelia were separated by filtration and both were extracted three times with ethyl acetate (broth/solvent was 3:1, v/v). After solvent evaporation the crude extract was fractionated on a Si-60 column (Lobar, Merck) by washing with *n*-hexane and a subsequent gradient of *n*-hexane/ethyl acetate changing from 95/5 to 1/1. When necessary the collected fractions were purified further by preparative TLC.

Instruments used: NMR: The ^{1}H NMR spectra were obtained at 400 MHz on a Bruker WM 400 spectrometer and the ^{13}C NMR spectra at 75.5 MHz on a Bruker AM 300 spectrometer, $CDCl_3$ was the solvent and TMS the internal standard. IR: spectra were measured in chloroform on a Perkin Elmer IR Spectral-Photometer 297. Mass spectra were recorded on an AEI 902S mass spectrometer with 70 eV. TLC solvent: *n*-hexane/ethyl acetate 1 + 1 = A.

Fermentation of 200 mg ferulic acid (**1**) with *Bacillus pumilus* DSM 492 yielded 155 mg 4-hydroxy-3-methoxystyrene [7786-61-0] (**1a**) after 22 h.

4-Hydroxy-3-methoxystyrene (**1a**): R_f (A): 0.50, ^{1}H NMR ($CDCl_3$): 6.94 (1H, d, J = 2 Hz, 2-H), 6.90 (1 h, dd, J = 8, 2 Hz, 6-H), 6.87 (1H, d, J = 8 Hz, 5-H), 6.62 (1H, dd, J = 18, 11 Hz, 7-H), 5.57 (1H, d, J = 18 Hz, 8-H), 5.11 (1H, d, J = 11 Hz, 8′-H), 3.86 (3H, s, OCH_3).

Fermentation of 200 mg isoferulic acid (**2**) with *Fusarium solani* DSM 63413 resulted in 110 mg 3-hydroxy-4-methoxystyrene [621-58-9] (**2a**) after 24 h.

3-Hydroxy-4-methoxystyrene (**2a**): R_f (A): 0.49, ^{1}H NMR ($CDCl_3$): 7.04 (1H, d, J = 2 Hz, 2-H), 6.78 (1H, d, J = 8 Hz, 5-H), 6.86 (1H, dd, J = 8, 2 Hz, 6-H), 6.61 (1H, dd, J = 17, 10 Hz, 7-H), 5.59 (1H, d, J = 17 Hz, 8-H), 5.12 (1H, d, J = 10 Hz, 8′-H), 3.88 (3H, s, OCH_3).

200 mg *p*-coumaric acid (**3**) were transformed by *Hansenula capsulata* DSM 70269 after 24 h to 150 mg 4-hydroxystyrene [2628-17-3] (**3a**).

4-Hydroxystyrene (**3a**): R_f (A): 0.52.

Hansenula capsulata DSM 70269 decarboxylated 200 mg caffeic acid (**5**) in 41 h to 78 mg 3,4-dihydroxystyrene [6053-02-7] (**5a**).

3,4-Dihydroxystyrene (**5a**): R_f (A): 0.33, ^{1}H NMR ($CDCl_3$): 6.93 (1H, d, J = 2 Hz, 2-H), 6.76 (2H, m, 5-, 6-H), 6.57 (1H, dd, J = 17, 10 Hz, 7-H), 5.53 (1H, d, J = 17 Hz, 8-H), 5.04 (1H, d, J = 10 Hz, 8′-H).

Fermentation of 200 mg 3,4-dimethoxy-cinnamic acid (**7**) yielded with *Hansenula anomala* DSM 70130 after 48 h 40 mg of 3,4-dimethoxystyrene [6380-23-0] (**7a**).

Table I: ^{13}C NMR data of **1a**, **2a**, **5a**, and **7a** (75.5 MHz, $CDCl_3$).

	1a	**2a**	**5a**	**7a**
C-1	130.1 0[a]	131.6 0	130.0 0	130.8 0
C-2	108.2 +	110.6 +	112.2 +	108.6 +
C-3	146.6 0	145.8 0	n.d.	149.0 0
C-4	145.7 0	146.5 0	n.d.	149.0 0
C-5	114.4 +	111.8 +	114.7 +	111.1 +
C-6	120.1 +	118.8 +	118.3 +	119.5 +
C-7	136.7 +	136.4 +	136.3 +	136.5 +
C-8	111.4 −	112.1 −	109.9 −	111.8 −
OCH_3	55.9 +	56.0 +	−	56.0 +
	−	−	−	55.9 +

[a] Amplitude of signals in DEPT-135 spectrum (CH_3 or CH = +; CH_2 = −; quat. C = 0).

3,4-Dimethoxystyrene (**7a**): R_f (A): 0.53, ^{1}H NMR ($CDCl_3$): (1H, d, J = 2 Hz, 2-H), 6.94 (1H, dd, J = 8 Hz, 6-H), 6.83 (1H, d, J = 8 Hz, 5-H), 6.66 (1H, dd, J = 17, 10 Hz, 7-H), 5.62 (1H, d, J = 17 Hz, 8-H), 5.15 (1H, d, J = 10 Hz, 8′-H), 3.91 (3H, s, OCH_3), 3.89 (3H, s, OCH_3).

200 mg *p*-methoxy-cinnamic acid (**11**) reacted with *Candida intermedia* DSM 70753 after 139 h to 25 mg *p*-methoxystyrene [637-69-4] (**11a**).

Candida intermedia DSM 70753 transformed 200 mg *o*-coumaric acid (**13**) within 139 h to 60 mg 2-hydroxystyrene [695-84-1] (**13a**).

Fermentation of 200 mg *m*-coumaric acid (**14**) with *Hansenula anomala* DSM 70130 resulted in 90 mg of 3-hydroxystyrene [620-18-8] (**14a**) after 69 h.

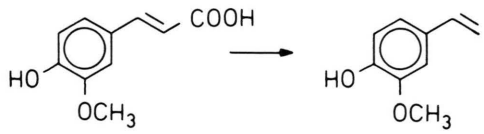
Results

Hydroxy-methoxy-substituted cinnamic acids

When ferulic acid (**1**) (4-hydroxy-3-methoxy-cinnamic acid) was incubated with *Bacillus pumilus* DSM 492 for 22 h only one reaction product was obtained in 96% yield, the respective styrene (**1a**). Beside this organism we found five others which gave

only the decarboxylation product **1a** in high yields. These results are depicted in Fig. 1.

Fig. 1. Decarboxylation of ferulic acid by various microorganisms.



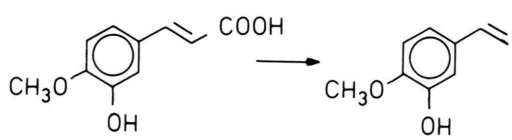
1 1a

Microorganisms	Yield [%]	Time [h]
Aspergillus carneus DSM 1518	48.5	118
Bacillus pumilus DSM 492	96.2	22
Candida intermedia DSM 70753	69.2	17
Fusarium moniliforme DSM 840	50.7	20
Fusarium oxysporum DSM 62287	88.9	48
Fusarium solani DSM 62413	61.4	48

Scaling up of the reaction by incubating 15 g of **1** with *Candida intermedia* DSM 70753 in a 20 l medium containing stirred fermenter it resulted after 69 h in 69% of **1a** exclusively. During our investigations on various other biotransformation reactions we found a lot of microorganisms which were able to decarboxylate ferulic acid in good yields, without resulting in further products. These organisms are listed in Table II. All these decarboxylation reactions were complete within 20 to 48 h.

The isomer of **1**, 4-methoxy-3-hydroxy-cinnamic acid (**2**), subjected to biotransformation with *C. intermedia* DSM 70753 ended after 24 h with the respective styrene **2a** in 51% yield. Fermentation with two *Fusarium* strains resulted also in the styrene with yields between 50 and 70%, as shown in Fig. 2.

Fig. 2. Yields for the decarboxylation of isoferulic acid.



2 2a

Microorganisms	Yield [%]	Time [h]
Candida intermedia DSM 70753	51.0	24
Fusarium oxysporum DSM 62287	54.3	24
Fusarium solani DSM 62413	71.1	24

Table II. Microorganisms which are able to decarboxylate ferulic acid in good yields.

Aspergillus carneus DSM 1518
A. ochraceus DSM 824
A. ochraceus NRRL 405
A. terreus DSM 62071
A. terreus DSM 826
Bacillus pumilus DSM 361
B. pumilus DSM 27
Candida intermedia DSM 70753
Corynespora cassiicola DSM 62474
Curvularia affinis DSM 63274
C. clavata DSM 62480
C. lunata DSM 63137
C. lunata ATCC 12017
C. lunata ATCC 13432
C. lunata ATCC 13633
Fusarium coerulum DSM 62178
F. dimerum DSM 62197
F. eumartii DSM 62213
F. moniliforme DSM 764
F. moniliforme DSM 840
F. oxysporum f. *aechmeae* DSM 62297
F. oxysporum DSM 62287
F. oxysporum ATCC 7808
F. oxysporum DSM 62291
F. oxysporum f. sp. *pisi* ATCC 9991
F. roseum DSM 3019
F. solani DSM 62413
F. solani DSM 62416
F. solani DSM 1164
F. solani var. *pisi* DSM 62420
F. tritinctum DSM 62446
Hansenula anomala DSM 70130
H. anomala AM 48
H. beckii DSM 70266
H. capsulata DSM 70269
H. henricii DSM 70272
H. minuta DSM 70274
H. saturnus DSM 70278
Saccharomyces cerevisiae CBS 1505
S. cerevisiae CBS 1508
S. cerevisiae v. *ellipsoides* DSM 70486

Hydroxy-substituted cinnamic acids

Fermentation of caffeic acid (**5**) with *C. intermedia* DSM 70753 was complete after 41 h and gave only the corresponding styrene **5a** in 22% yield. A second organism, *Hansenula capsulata* DSM 70269, was also able to convert **5** to **5a** in 41 h with 51.6% yield (Fig. 3). *o*-Coumaric acid (**13**) could be decarboxylated by *C. intermedia* DSM 70753 within 139 h to **13a** in 41% yield. No further microorganisms were found which could perform this reaction.

When *m*-coumaric acid (**14**) was subjected to transformation with *Hansenula anomala* DSM 70130, the styrene **14a** was obtained after 69 h in

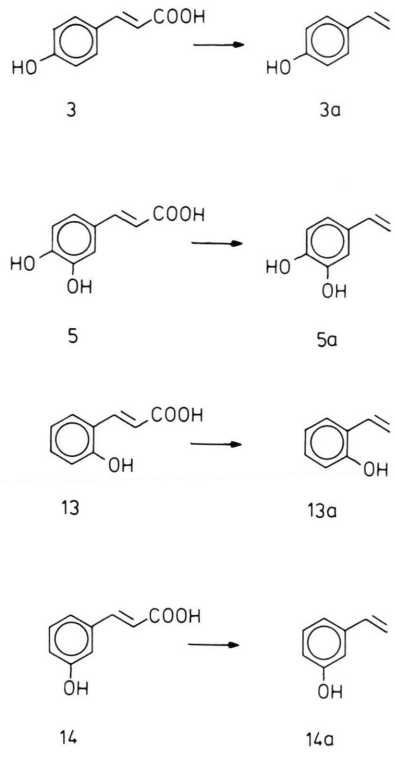


Fig. 3. Hydroxy- and dihydroxycinnamic acids which could be decarboxylated by microorganisms.

61% yield. With *C. intermedia* DSM 70753 **14a** was obtained only in very low yield.

Biotransformation with *p*-coumaric acid (**3**) revealed three microorganisms by which the compound could be transformed to the styrene **3a**. *Hansenula capsulata* DSM 70269 gave 97.4% **3a** within 24 h, *Curvularia fallax* DSM 63169 gave 34% within 21 h, and *C. intermedia* DSM 70753 afforded after 24 h 57.8% of **3a**.

Methoxy-substituted cinnamic acids

After incubation of 3,4-dimethoxy-cinnamic acid (**7**) for 48 h with *Hansenula anomala* DSM 70130 the styrene **7a** was obtained in 25.4% yield. No further organisms could be found which are able to decarboxylate that compound in desirable yields.

4-Methoxy cinnamic acid (**11**) also could only be decarboxylated with one organism, namely *C. intermedia* DSM 70753. After 45 h it resulted in 16.6% of **11a** (Fig. 4).

For further methoxy-substituted derivatives like 2-methoxy-, 3-methoxy-, 2,5-dimethoxy-, and 3,5-dimethoxy-cinnamic acid no microorganisms could be found which were able to carry out the decarboxylation reaction.

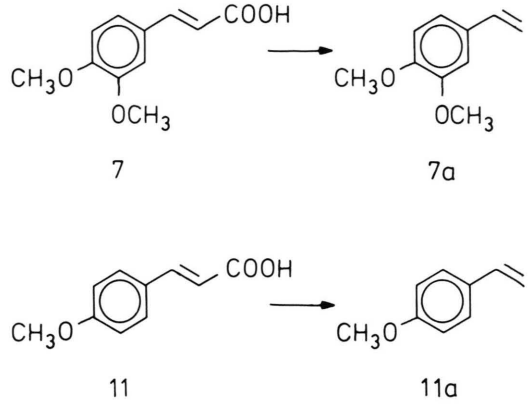
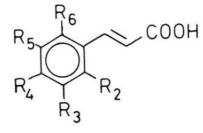


Fig. 4. Decarboxylation of methoxy derivatives of cinnamic acids.

Methyl- and halo-substituted cinnamic acids

All compounds which have been subjected to biotransformation to obtain the respective styrenes are listed in Table III.

Table III. Compounds which were subjected to microbial decarboxylation.



	R_2	R_3	R_4	R_5	R_6
1	H	OCH_3	OH	H	H
2	H	OH	OCH_3	H	H
3	H	H	OH	H	H
4	H	OCH_3	H	OCH_3	H
5	H	OH	OH	H	H
6	H	OCH_3	OH	OCH_3	H
7	H	OCH_3	OCH_3	H	H
8	H	H	H	H	H
9	OCH_3	H	H	H	H
10	H	H	OCH_3	OCH_3	H
11	H	H	OCH_3	H	H
12	H	OCH_3	H	H	H
13	OH	H	H	H	H
14	H	OH	H	H	H
15	H	H	CH_3	H	H
16	Cl	H	H	H	H
17	H	Cl	H	H	H
18	H	H	Cl	H	H
19	Cl	H	H	H	Cl
20	H	H	Br	H	H

All microorganisms tested with the methyl- and different halo-substituted cinnamic acids were not able to transform these compounds to the corresponding styrenes. It did not occur any reaction at all, the compounds remained unchanged during the fermentation process.

Aromatic carboxylic acids with saturated side chains

All microorganisms which exhibited the ability to decarboxylate the cinnamic acid derivatives were also tested with various aromatic carboxylic acids with saturated side chains varying in their length from formic acid to butyric acid. Carboxylic acids with indole as the aromatic part of the molecule were included, too. All phenyl compounds were also tested as the same substitution pattern as the cinnamic acids.

But none of these substances could be decarboxylated by the microorganisms active at the cinnamic acid derivatives. Only one exception was found with *Corynespora cassiicola* DSM 62474 which was able to decarboxylate salicylic acid in 30% yield within 67 h to phenol. But 3-hydroxy-, 4-hydroxybenzoic acid and vanillic acid could not be decarboxylated by this fungus.

In this context it should be mentioned that unsubstituted cinnamic acid was also resistant to any decarboxylation reaction by the microorganisms used.

Discussion

The microbial decarboxylation of substituted cinnamic acids resulted in high yields of the respective styrenes. All reactions were completed within 24 to 48 h and showed no side products. Especially ferulic acid could be transformed in almost quantitative yields. The resulting styrene is a compound used in fragrance and perfume industry. The chemical synthesis of that styrene proceeds in yields of 50 to 60% [12, 13]. The amount of styrene decreased a little in the case of isoferulic acid, when the two ring substituents were interchanged. Both compounds, ferulic acid and isoferulic acid have been decarboxylated by an isolated enzyme from an *Aerobacter* strain, but no yields were given [2]. The resulting styrenes are occurring in nature, they were identified in extracts of leaves of Kudzu plants [13].

The *o*-, *m*-, and *p*-coumaric acids were also good substrates for the microorganisms to be decarboxylated in 40–97% yield whereby the yields decreased in the sequence *p*-, *m*-, and *o*-substitution. Compared to the chemical synthesis of the respective vinyl phenols with yields of about 40% the biotransformation can well compete [14, 15]. A microbial decarboxylation of the coumaric acids was obtained under aerobic and anaerobic conditions with a *Bacillus* strain isolated from rat intestine, but no yields were determined [16]. For the decarboxylation of 3,4-dihydroxy-cinnamic acid we got the styrene in 20–50% yield. Two examples are known in literature for a microbial decarboxylation of 3,4-dihydroxy-cinnamic acid, one with the *Bacillus* strain [16] and one under resting cell conditions with *Polyporus circinata* NRRL 2903 [17], but no yields were described. The 3,4-dihydroxy styrene was recently isolated from a culture broth of the fungus *Fomes tasmanicus* and was shown to be an inhibitor for phenylalanine hydroxylase [18].

The methoxy- and dimethoxy derivatives of cinnamic acid gave much lower yields in the microbial decarboxylation reaction than the hydroxy derivatives and there are only few microorganisms which could performe the reaction. For *Saccharomyces cerevisiae* a decarboxylation of 3,4-dimethoxy-cinnamic acid was described to yield 62% styrene [3], but with the strains used by us we could not get any decarboxylation.

The benzoic acid derivatives failed to react with the microorganisms to the resulting phenols with the exception of salicylic acid which could be decarboxylated in 30% yield. But the same fungus did not react with vanillic acid and other hydroxybenzoic acids. From the literature are known two examples of decarboxylation of benzoic acid derivatives by microorganisms or enzymes thereof. Vanillic acid was transformed to vinylguaiacol by *Bacillus megaterium* strains isolated from soil and a *Streptomyces* strain [19], and an enzyme isolated from *Aspergillus niger* decarboxylated 3,4-dihydroxybenzoic acid [20]. Despite the fact that several publications appeared on microbial decarboxylation, we could show for the first time in preparative scales that substituted styrenes can be obtained in very good yields without any side products from the respective cinnamic acids by microorganisms. This method can well compete with chemical synthesis and in some cases is even superior. A prerequisite for the microbial synthesis of substituted styrenes from cinnamic acids is a substitution of the aromatic ring at the 3- and/or 4-position with an oxygen containing group. Substituents

without oxygen, like methyl- or halo-groups or unsubstituted cinnamic acid could not be decarboxylated with the strains we tested. The microorganisms also failed to react with the compounds when the 5-ring position is substituted (see Table III).

Acknowledgements

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