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Selective hydrolysis of the nitrile group of *cis*-dihydrodiols from aromatic nitriles

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Abstract

Several nitrilases were screened for the hydrolysis of the nitrile group of *cis*-1,2-dihydroxy-3-cyanocyclohexa-3,5-diene and *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile to the corresponding acids. Nitrilase from *Rhodococcus* sp. was able to convert both compounds with the activity of 0.3 mU/mg protein and 0.05 mU/mg protein, respectively. Nitrilase AtNIT1 from *Arabidopsis thaliana* converted only the latter but with a higher initial specific activity of 1.7 mU/mg protein. Biotransformation of *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile was performed with AtNIT1 in the form of isolated enzyme and immobilized enzyme, and with recombinant cells containing AtNIT1. Biotransformations with isolated AtNIT1 resulted in 116 mg of product in 10.6 h with a yield of 77%. Forty-three percent of the enzymatic activity could be recovered after the biotransformation. Immobilization of AtNIT1 saturated with 3-phenylpropionitrile resulted in 3.5% of the free enzyme activity. Biotransformations with *Escherischia coli* JM101 (pQE10-AtNIT1) in shake-flasks produced 243 mg of product in 23 h with a yield of 48%. Maximum and average specific activities of 0.5 U/g cell dry weight and 0.17 U/g cell dry weight were achieved, respectively.

Keywords: Biocatalysis; Biotransformations; Dihydrodiols; Nitrile hydrolysis; AtNIT1

1. Introduction

Enzymes are powerful catalysts with several attractive features including substrate selectivity, chemo-, regio-, and enantioselectivity [1]. These features enhance application of biocatalysts in chemical synthesis [2] and provide opportunities for biocatalytic steps in asymmetric catalysis on an industrial scale [3]. Use of biocatalysis becomes especially interesting for the production of compounds, for which chemical synthesis is not a practical option or the required regioselectivities or stereoselectivities are not achieved [4], such as *cis*-dihydroxylation of aromatic compounds by bacterial dioxygenases [5]. Among the *cis*-arene diols identified [5–7] *cis*-dihydrodiols with a nitrile functionality are especially interesting, since the nitrile group can be hydrolyzed to form a new class of acidic chiral diols. Chemical hydrolysis of the nitrile group requires harsh conditions, such as heating at an acidic or alkaline pH [8]. This hinders the selective transformation, since the *cis*-dihydrodiols dehydrate to the corresponding phenols under these conditions [9]. On the other hand, biocatalysis offers chemoselective hydrolysis at neutral pH and room temperature. Nitrile groups can be hydrolyzed selectively by enzymes along two distinct routes (Fig. 1): direct hydrolysis of the nitrile group to an acid by a nitrilase, or hydration of the nitrile by a nitrile hydratase to an amide, which can then be hydrolyzed to the corresponding acids by an amidase [10].

In our previous work, we showed that *Escherischia coli* JM101 (pTEZ30) carrying chlorobenzene dioxygenase (CDO) of *Pseudomonas* sp. strain P51 [11] catalyzes several aromatic nitriles to the corresponding *cis*-dihydrodiols [12,13]. In this paper, we describe the results of a screening aimed for a nitrilase that hydrolyzes the nitrile group of *cis*-1,2-

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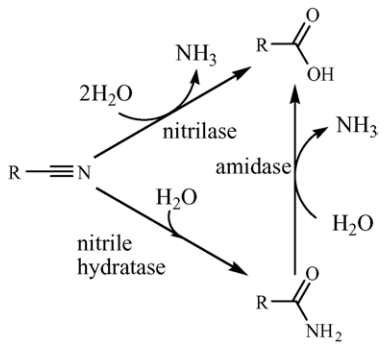

Fig. 1. Pathways of enzyme-catalyzed hydrolysis of nitriles.

dihydroxy-3-cyanocyclohexa-3,5-diene (**2**) and *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile (**6**) (Fig. 2). Furthermore, hydrolysis reactions with isolated and immobilized enzymes and with recombinant cells carrying the nitrilase are compared and discussed with respect to their efficiency and practical applicability.

2. Experimental

2.1. Chemicals and buffers

Chemicals with the following purities were used: benzonitrile, >99%; *trans*-cinnamonitrile, >99%; benzoic acid, >99%; 2-hydroxybenzoic acid, >99%; 3-hydroxybenzoic acid, >99%; 3-phenylpropionitrile, >98% (Fluka AG, Buchs, Switzerland); 2-hydroxycinnamic acid, 97%; 3-hydroxycinnamic acid, 99%; 4-hydroxycinnamic acid, 98%; cinnamic acid, >99%; 4-hydroxybenzoic acid, >99%; (Aldrich), *cis*-1,2-dihydroxy-3-cyanocyclohexa-3,5-diene and *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile were prepared in our earlier studies [12,13]. All buffers were prepared as recommended by the enzyme supplier (Fluka Chemie AG, Buchs) and according to the standard protocols [14].

2.2. Enzymes

Nitrilase (EC Number: 3.5.5.1, CAS Number: 9024-90-2) in lyophilized form was supplied from Fluka Chemie AG, Buchs. All enzymes were kept under argon and stored at −20 °C. Nitrilase from *Alcaligenes faecalis*, BioChemika, powder, slightly red, ∼15 U/g towards 3-phenylpropionitrile at pH 7.5 and 30 °C [15]; nitrilase from *Arabidopsis thaliana* (AtNIT1), recombinant from *E. coli*, 480 kDa, 11–13 subunits BioChemika, powder, faintly beige 0.3–1.0 U/mg towards 3-phenylpropionitrile at pH 8 and 35 °C [16–19]; nitrilase 1 from *Pseudomonas fluorescens*, 78 kDa, 2 subunits, BioChemika powder, slightly yellow, 10–15 U/g towards benzonitrile at pH 7.5 and 30 °C [20]; nitrilase 2 from *P. fluorescens*, 78 kDa, 2 subunits, BioChemika, powder, 10–15 U/g towards benzonitrile at pH 7.5 and 30 °C [20]; nitrilase from *Rhodococcus rhodochrous*, BioChemika, powder, slightly beige, ∼10 U/g towards benzonitrile at pH 8 and 30 °C [21,22]; nitrilase from *Rhodococcus* sp., 560 kDa, 12 subunits, BioChemika, powder, slightly red, >0.1 U/mg towards benzonitrile at pH 7.5 and 30 °C; nitrilase from *Pseudomonas putida*, BioChemika, powder pH 7.5 and 30 °C. Activity tests were performed with lyophilized enzymes under the conditions given by the supplier. For biotransformations lyophilized AtNIT1 or dialyzed AtNIT1 was used. The protein amounts of the lyophilized nitrilases and the dialyzed AtNIT1 were determined with Bradford method [23]. The amount of enzyme (a subunit mass of 38 kDa) [24] was estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the standard methods [14].

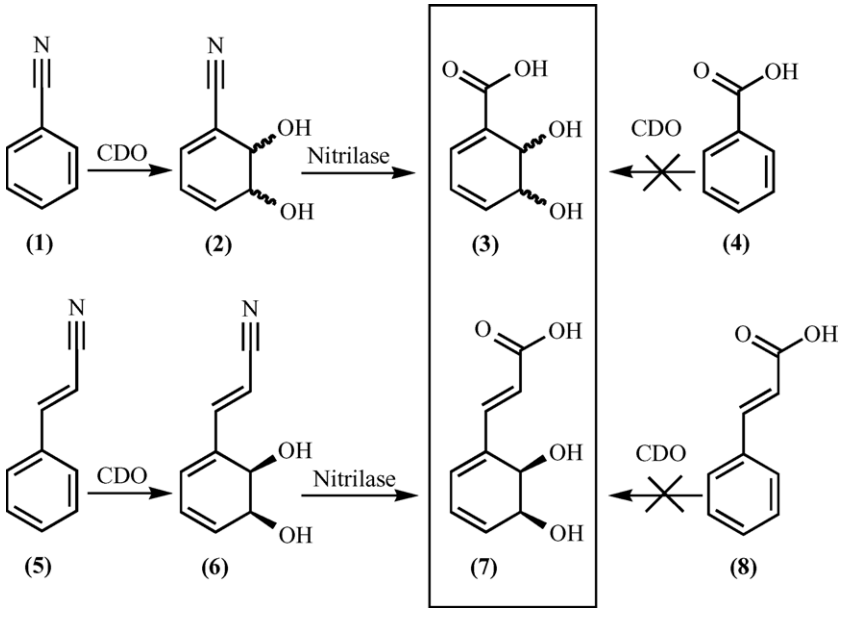

Fig. 2. *cis*-Dihydroxylation of benzonitrile (**1**) and cinnamonitrile (**5**) with chlorobenzene dioxygenase followed by the hydrolysis of the nitrile group with a nitrilase.

2.3. AtNIT1 nitrilase preparation

Twenty-five millilitres preculture medium containing 10 g/L tryptone (Fluka AG, Buchs, Switzerland), 5 g/L yeast extract (Fluka AG, Buchs, Switzerland), 10 g/L sodium chloride, 50 mg/L ampicillin, and 20 mg/L kanamycin was inoculated with *E. coli* E16M15 (pQE10-AtNIT1) and incubated at 37 °C on a horizontal shaker at 160 rpm for 1 day. Six hundred millilitres fresh medium was inoculated with this preculture and incubated under the same conditions as the first preculture. Bioreactors containing 12-L of the same medium were inoculated with the second preculture and fermentation was performed at pH 7.5 and at 30 °C. Induction was started with the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG), whereby the temperature was decreased to 20 °C or to 30 °C. At the end of the fermentation, the culture was centrifuged at 4 °C at 14,000 × *g* for 10 min. The cell pellet was homogenized in a Dynomill Beadmill (Bachofen, Basel, Switzerland) with 0.1–0.2 mm glass beads in 0.5 mM EDTA and 0.5 mM dithiothreitol (DTT) at pH 8.0 and 4 °C for 30 min. The homogenate was centrifuged at 4 °C at 14,000 × *g* for 5 min and the supernatant was simultaneously ultrafiltered and concentrated with a Millipore Pellicon Ultrafiltration system using polyethersulfon membranes (P2B010A05) with a MWCO of 10 kD in a Pellicon 2 cassette and an area of 2 m × 0.5 m against 0.5 mM EDTA and 0.5 mM DTT and at pH 8.0. The concentrated retentate corresponding to a purified dialyzate is then frozen at −80 °C and lyophilized. Nitrilase activities have been determined by measuring the released ammonia when 3-phenylpropionitrile was used as a substrate at pH 8.0 and at 35 °C. One unit (U) corresponds to the 1 μmol of ammonia formed in 1 min at pH 8 and 35 °C in the hydrolysis of 3-phenylpropionitrile to 3-phenylpropionic acid.

2.4. Biotransformation of benzoic acid and cinnamic acid

Biotransformation of benzoic acid and cinnamic acid was performed in shake-flasks with *E. coli* JM101 (pTEZ30) carrying CDO as described elsewhere [12]. To start the biotransformation 2 mM substrate was added directly. Samples were taken at different time points, diluted two-fold with ice-cold acetonitrile and centrifuged at 4 °C at 20,000 × *g* for 6 min. Supernatants were filtered through a cellulose membrane filter (Spartan, Schleicher & Schuell GmbH, Germany) and analyzed via HPLC and HPLC-MS.

2.5. Activity assays

All activity measurements were performed in a shaker (Thermomixer 5436, Eppendorf) with 600 rpm. Two millilitres Eppendorf tubes were used for the incubation of enzyme and substrate solutions. Activity tests of nitrilases were performed under assay conditions given by the supplier. Substrates and enzymes were dissolved separately in buffer and incubated for 1 h at assay temperature. The reaction was started by the addition of the substrate solution to the enzyme solution. The reactions were followed for 150 min. Samples were taken at different time points, diluted 1:1 with acetonitrile, centrifuged at room temperature and at 20,000 × *g* for 8 min. Supernatants were analyzed for product formation by HPLC and HPLC-MS. At the end of the reaction, protein concentrations were verified and pH values were checked. One unit (U) is defined as 1 μmol of product formed in 1 min.

2.6. HPLC analysis

HPLC measurements were performed on a HPLC-system consisting of a 1040 M SERIES II Diode-array detection and a Supersphere 100 RP-18/LiChroCART 125-4 HPLC Cartridge (Merck, Germany) as a stationary phase. The elution pattern was monitored at 210 nm, 254 nm (aromatic systems), 290 nm (maximum absorbance of (**2**) and (**3**)), and 320 nm (maximum absorbance of (**6**) and (**7**)). Two different mobile phase have been used: (a) 100% nanopure water containing 0.1% *o*-phosphoric acid for the analysis of samples containing compounds (**2**) and (**3**) and (b) 95.5% nanopure water containing 0.1% *o*-phosphoric acid and 4.5% acetonitrile for the analysis of samples containing compounds (**6**) and (**7**) with an isocratic flow rate of 0.8 mL/min. These methods allowed the clear separation of substrates and products with the following retention times: (**3**) 5 min; (**2**) 10.5 min; (**7**) 9.7 min; (**6**) 13 min.

2.7. HPLC-MS analysis

Reversed phase HPLC-MS analysis was performed on a HEWLETT PACKARD SERIES 1100/MSD system consisting of a degasser, a BinPump, a ColComp and a mass spectrum detector. The system was supplemented with a Multipurpose Sampler MPS2 (Gerstell). The mass spectrum detector was set to negative mode. Separation was performed on a nucleosil C18 RP column (pore size, 100 Å; particle size 5 μm; inner diameter, 12.5 cm × 2 mm Macherey-Nagel AG, Oensingen, Switzerland) with 85% nanopure water containing 0.1% formic acid and 15% acetonitrile as a mobile phase with a isocratic flow rate of 0.8 mL/min at a temperature of 45 °C.

2.8. Dehydration of cis-diols

The product (**7**) was dissolved in water, pH was reduced to 1 with 30% HCl. Then it was incubated at 90 °C for 90 min. The resulting compounds were analyzed with HPLC-MS.

2.9. Hydrolysis of (*6*) to (*7*) with isolated AtNIT1

Small-scale hydrolysis of (**6**) to (**7**) was carried out with lyophilized AtNIT1 at 0.8 and 2.6 mg protein/mL, with different substrate concentrations. For larger scale hydrolysis, 16.6 mL potassium phosphate (KPi) buffer containing 50 mM (**6**) and 50 mL of dialyzed AtNIT1 (221 units towards 3-PPN) were incubated separately on a shaker (35 °C, 85 rpm). After 1 h of incubation, 5.5 mL of buffer containing (**6**) was added to the enzyme solution. Samples were taken at different time points, diluted 1:1 with acetonitrile and centrifuged for 8 min at 14,000 rpm. HPLC analysis was used to follow product formation. Additional substrate containing buffer was added during the reaction; 3.5 mL

after 2.2 and 3.7 h, and 4.1 mL after 5.2 h. After 10.6 h, the solution was centrifuged at 20,000 × *g* and room temperature for 15 min. The supernatant was then transferred into a Millipore 10.000 MWCO tube and centrifuged at 4000 rpm at 4 °C until the concentrated protein fraction was slightly covered with a remaining liquid phase (corresponding to a volume of 1.5 mL). The protein fraction was then resuspended in KPi buffer to a final volume of 35 mL and stored at 4 °C. Protein amount was determined. The filtered solution was analyzed via HPLC to check the product concentration. The recovered protein solution was checked for activity towards 3-PPN. The activity was measured as in Section 2.5.

2.10. Immobilization of AtNIT1 on Eupergit® C and activity test of immobilized enzymes

The KPi immobilization buffer (pH 8, 1 M) was prepared in accordance with the supplier manual (Röhm, Darmstadt, Germany). A 33.4 mg of lyophilized AtNIT1 was dissolved in 5.5 mL immobilization buffer in a 50 mL Falcon tube and incubated at room temperature (RT) for 1 h. One gram of Eupergit® C was added and the solution (2.7 mg protein/mL/mg Eupergit® C) was shaken gently. The immobilization solution was stored at RT for 96 h. For immobilization of AtNIT1 on Eupergit® C in the presence of 3-phenylpropionitrile, 500 μL dialyzate was dissolved in 3.5 mL immobilization buffer in a 50 mL Falcon tube and incubated at 35 °C for 1 h. One millilitre 3-phenylpropionitrile (50 mM in immobilization buffer supplemented with 5% methanol, 35 °C) was added to the dialyzate solution and kept at 35 °C for 18 min prior to adding 0.9 g Eupergit® C. The immobilization solution (2.7 mg protein/mL/ mg Eupergit® C) was stored at 6 °C for 96 h. In each case the beads were collected on a sintered-glass filter (porosity 2) and the solutions drained off by vacuum. Fifty millilitres KPi buffer were used to wash the beads thoroughly on the filter. Protein concentrations in the filtered solution were measured with the Bradford method to determine the binding yield. The beads were finally dissolved in KPi buffer. All activity measurement experiments were performed on a shaker (Thermomixer 5436, Eppendorf, 600 rpm). Immobilized enzymes as well as substrate solutions were incubated in 2 mL Eppendorf tubes for 1 h at 35 °C prior determination of enzyme activities. Reactions were started with addition of substrate into bead-solution yielding to initial concentrations of 1.9 mM (**6**) or 2 mM 3-phenylpropionitrile and 0.1% methanol. Samples were taken at different time points, diluted 1:1 with acetonitrile, and centrifuged at RT and at 20,000 × *g* for 8 min. Supernatants were analyzed by HPLC.

2.11. Hydrolysis of (*6*) to (*7*) with E. coli JM101 (pQE10-AtNIT1)

For small scale hydrolysis experiments, the freshly transformed cells were plated out on LB agar plates supplemented with the antibiotics kanamycin and ampicillin and incubated overnight at 37 °C. The next day, a single colony was transferred into 5 mL LB medium with the appropriate antibiotics and incubated for 10 h at 37 °C and 250 rpm. One millilitre of the LB preculture was then transferred to 100 mL M9, supplemented with 150 μg/mL ampicillin and 50 μg/mL kanamycin medium and incubated overnight at 30 °C and 200 rpm. One hundred millilitres M9 medium were inoculated with this preculture to an OD_{450} of 0.35 and incubated on a shaker for half an hour. The culture was induced with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 h, and during this time the culture was grown to an OD_{450} of 1.36. At this time point, the cells were centrifuged (9700 × *g*, 4 °C, 15 min), and resuspended in 20 mL 0.1 M KPi buffer (pH 8.23) to an OD_{450} of 5.9. The reaction was started by the addition of (**6**) into the cell suspension and incubated at 30 °C and 130 rpm. As a positive control we tested the activity of the cells towards 3-PPN. Samples were taken at different time points and diluted 10 times with ice-cold acetonitrile and centrifuged at 20,000 × *g*, at 4 °C for 5 min. Supernatants were acidified to pH 1 with 30% HCl and incubated at 95 °C for 20 min. 2-Hydroxycinnamic acid and 3-hydroxycinnamic acid formed were analyzed with HPLC and quantified using the standards.

For larger scale hydrolysis experiments, cells were grown in a reactor with a working volume of 2-L and induced with 0.35 mM IPTG as described elsewhere [12]. After the induction, cells were harvested and washed with 5 mM KPi Buffer (pH 7.15) and stored at −80 °C in fractions. A fraction of the frozen cells was resuspended in 100 mM KPi buffer (pH 8.3) to a cell density of 50 g cdw/L. Substrate was added to the 114 mL total reaction medium and incubated at 35 °C on a rotary shaker at 250 rpm. Four hours later additional substrate was added and the reaction was followed for almost 23 h. Samples were taken at different time points and treated as described above.

3. Results and discussion

One approach for the synthesis of (**3**) and (**7**) is the *cis*-dihydroxylation of benzoic acid (**4**) and cinnamic acid (**8**) (Fig. 2). Thus, before starting the screening for a nitrilase, we tested the activity of *E. coli* JM101 (pTEZ30) carrying CDO towards these compounds. HPLC and HPLC-MS measurements of the samples of such biotransformation experiments did not reveal any product formation. In the literature, there is only one strain described that was able to *cis*-dihydroxylate substituted benzoic acids on an unsubstituted aromatic ring [25,26]. Other strains metabolize the substituted benzoic acids to 1,2-*cis*-dihydrodiols [27–36]. No report was found describing the *cis*-dihydroxylation of cinnamic acid by a bacterial dioxygenase.

Table 1 shows the results of the screening experiments. The nitrilases listed were screened for ability to hydrolyze the nitrile group of *cis*-1,2-dihydroxy-3-cyanocyclohexa-3,5-diene (**2**) and/or *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile (**6**). A nitrilase from *Rhodococcus* sp. was able to convert both (**2**) and (**6**) to the corresponding acids with a specific activity of 0.3 mU/mg protein and 0.05 mU/mg protein, respectively. AtNIT1 from *A. thaliana* converted only (**6**), but with a higher initial specific activity of 1.7 mU/mg protein. Hydrolysis of (**6**) with nitrilases from *A. faecalis* and *R.*

Table 1
Specific activity of the nitrilases on (**2**) and (**6**)

Nitrilase	Protein concentration (mg protein/mL)	Substrate			
		(**2**)		(**6**)	
		Concentration (mM)	Specific activity (mU/mg protein)	Concentration (mM)	Specific activity (mU/mg protein)
Alcaligenes faecalis	1	4.5	–	[a]	
Arabidopsis thaliana (AtNIT1)	2.6	3.6	–	1.7	1.7
Pseudomonas fluorescens, nitrilase 1	5.8	3.7	–	1.9	–
Pseudomonas fluorescens, nitrilase 2	5.8	3.9	–	2	–
Pseudomonas putida	9.9	3.8	–	1.5	–
Rhodococcus rhodochrous	1	12	–	[a]	
Rhodococcus sp.	2.3	3.5	0.3	1.9	0.05

(–) No product formation was observed.
[a] Not tested.

rhodochrous were not tested. Other nitrilases did not show any activity on (**2**) and/or (**6**). Activity of each enzyme towards its reference substrate was tested as a positive control and similar activities were observed as given by the supplier (results not shown).

Some nitrilases hydrolyze aromatic or heterocyclic nitriles to the corresponding acids and ammonia. Others are known to preferentially hydrolyze either aliphatic nitriles or arylacetonitriles to their respective carboxylic acids [37]. One of the reasons for the lack of the activity of some nitrilases towards the substrates (**2**) and (**6**) might be steric hindrance of the two hydroxyl groups at *ortho* and *meta* positions [38]. For instance, all monosubstituted phenylacetonitriles are good substrates for the nitrilase from *A. faecalis* JM3, especially *p*-fluoro- and *p*-chloro-benzylcyanide. However, the activity dramatically decreases with substrates that carry the substitution at an *ortho* position instead of the *para* position [15]. In contrast to other nitrile-hydrolyzing enzymes, AtNIT1 has a broad substrate range [24]. The activity of AtNIT1 towards only (**6**) and not (**2**) is expected as it has 18 times higher specific activities towards cinnamonitrile than towards benzonitrile [24].

Hydrolysis products (**3**) and (**7**) were characterized with HPLC-MS. Mass fragments are presented in Table 2. All spectra were recorded under negative mode and consequently the deprotonated molecules (M_w −1 g/mol) were detected. Mass spectra showed that the product formed from hydrolysis of (**2**) by nitrilase from *Rhodococcus* sp. is the corresponding acid (**3**), and the product formed from hydrolysis of (**6**) by nitrilase from *Rhodococcus* sp. or AtNIT1 is the corresponding acid (**7**). AtNIT1-catalyzed hydrolysis may also result in amide formation depending on the nature of the substituents [24]. HPLC-MS measurements of the hydrolysis products of (**2**) and (**6**) did not show amide formation during biotransformation.

cis-Dihydrodiols dehydrate to the corresponding phenols under acidic conditions and high temperatures [39]. Dehydration products of (**7**) were analyzed with HPLC-MS. The results showed that the dehydration of (**7**) resulted in 2-hydroxycinnamic acid (83%) and 3-hydroxycinnamic acid (17%), confirming the structure of (**7**).

Next to the catalyst selectivity, process efficiency depends on turnover frequency, total turnover number, and volumetric productivity [40]. To investigate the practical applicability of the hydrolysis process, we examined the hydrolysis of (**6**) to (**7**) with AtNIT1 in the form of isolated and immobilized enzymes, and with *E. coli* JM101 (pQE10-AtNIT1 [24]) carrying AtNIT1.

First, hydrolysis with isolated AtNIT1 was further studied with different enzyme and substrate concentrations (Fig. 3). Product formation was followed for 150 min. Initial specific activity of AtNIT1 at low protein concentrations and reached a maximum of 3.2 mU/mg protein with 0.8 mg protein/mL of enzyme and 0.45 mM substrate. When a protein concentration

Table 2
The mass spectra of the products and substrates

Compound, M_w (g/mol)	Fragments (*m*/*z*)
(**2**), 137	136, 118 (–H_2O)
(**3**), 156	155, 137 (–H_2O)
(**6**), 163	162, 144 (–H_2O), 136 (–CN)
(**7**), 182	181, 163 (–H_2O), 119 (–CO_2–H_2O), 111 (–$O_2C_3H_4$)

Mass spectra were recorded in negative mode.

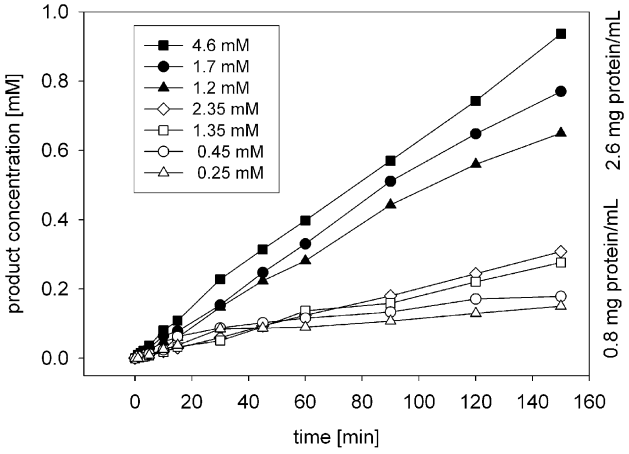

Fig. 3. Hydrolysis of *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile to the corresponding acid with AtNIT1 from *Arabidopsis thaliana*. 2.6 mg protein/mL and 0.8 mg protein/mL were used with different substrate concentrations.

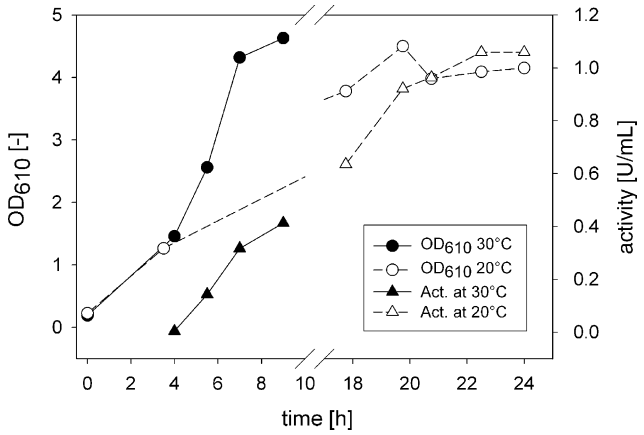

Fig. 4. Large scale AtNIT1 nitrilase preparation. Fermentation of *E. coli* E16M15 (pQE10-AtNIT1) was performed in a bioreactor on a 12-L scale at 20 °C and 30 °C.

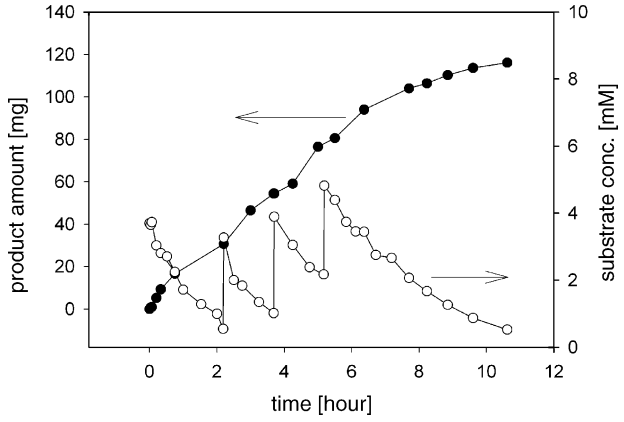

Fig. 5. Hydrolysis of *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile to the corresponding acid with isolated AtNIT1 from *Arabidopsis thaliana*. Reaction was started with the addition of substrate to 50 mL AtNIT dialyzate (300 units). Additional substrate was added at time 2.2, 3.7 and 5.2 h.

of 2.6 mg protein/mL was used, initial specific activity was lower (around 2 mU/mg protein) but product formation was more stable over the whole bioconversion. Productivity became more stable with increasing initial substrate concentrations.

We performed the fermentation of *E. coli* JM101 (pQE10-AtNIT1) in a bioreactor on a 12-L scale to prepare AtNIT1 nitrilase on large scale and to use it for further in vitro experiments. Growth of the recombinant cells and specific activities of the AtNIT1 nitrilase were followed during the fermentation at 30 and 20 °C (Fig. 4). Specific growth rate was higher at 30 °C and OD_{610} reached to 4.6 after 9 h. At the end of the fermentation volumetric activity increased to 0.41 U/mL. At 20 °C growth rate was lower and cell density reached to OD_{610} 4.2 after 24 h. The volumetric activity of 1.06 U/mL obtained at the end of the fermentation is significantly higher than that obtained at 30 °C. Total activities and purification yields are listed in Table 3.

To examine the specific activity and stability of the isolated AtNIT1 on a larger scale, and the effect of higher product concentrations on enzymatic activity, hydrolysis of (**6**) was performed using 221 units (towards 3-PPN) of AtNIT1 for 10.6 h. Almost 116 mg of product was formed with a yield of 77% (Fig. 5). Specific activity was between 3 and 4 mU/mg protein after each addition of substrate and was stable over the reaction time. The activity decreased with decreasing substrate concentration (Fig. 6). Maximum specific activity is in the same range as the initial activity of the AtNIT1 in small scale experiments. The slightly change in the activity might be due to different AtNIT1 content of the protein used. Enzyme activity was not affected by product concentrations up to 9.6 mM. After the biotransformation, 43% of the total nitrilase activity could be recovered. Ammonium sulphate precipitation or different membrane separations may further increase the recovery of activity.

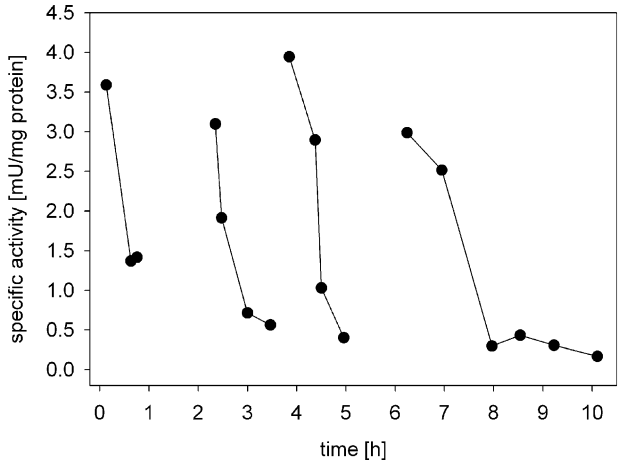

Fig. 6. Specific activity profile during the hydrolysis of *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile with isolated AtNIT1 from *Arabidopsis thaliana*. Substrate was added at time 0, 2.2, 3.7 and 5.2 h.

Second, hydrolysis of (**6**) with immobilized AtNIT1 was studied. For this purpose AtNIT1 was immobilized on Eupergit® C with 98% of the protein bounded to the resin. However, the immobilized enzymes did not show any activity with either (**6**) or 3-PPN as the substrate. One reason for this enzyme inactivity might be that the catalytic cystine groups of the enzymes reacted with the oxiran groups of Eupergit® C. Alternatively, the multimeric active structure of the enzyme complex might be lost during the binding process [24,41]. Therefore, to protect the catalytic center of the AtNIT1 [41], enzyme was saturated with 3-phenylpropionitrile and then immobilized on Eupergit® C with a yield of 79%. Hydrolysis of 3-PPN with the immobilized enzymes resulted in an activity of 8.4 mU/mg protein,

Table 3
Purification of AtNIT1-nitrilase from the fermentation of *E. coli* E16M15 (pQE10-AtNIT1) performed in a bioreactor on a 12-L scale at 30 °C

Fraction	Total mass (g)	Total activity (U)	Specific activity (U/mg)	Purification fold	Purification yield (%)
Biomass	533	13200	0.025	1	100
Lyophilized material	27.4	7820	0.286	11	59

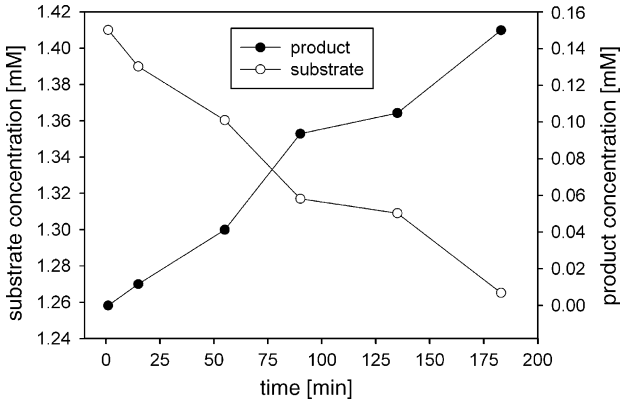

Fig. 7. Hydrolysis of *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile to the corresponding acid with *E. coli* JM101 (pQE10-AtNIT1). A 1.4 mM substrate was added to 20 mL 0.1 M KPi buffer (pH 8.23) containing 1.7 g of cell dry weight/L.

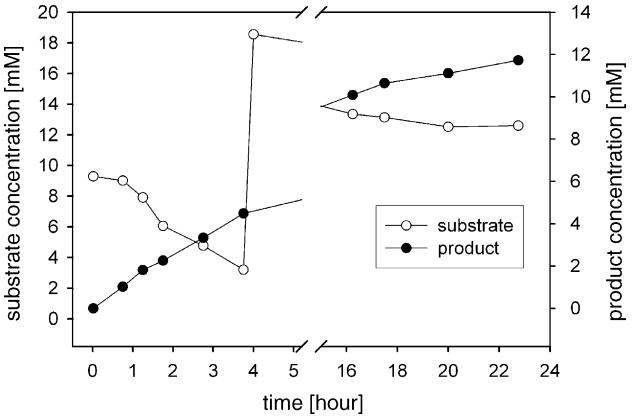

Fig. 8. Hydrolysis of *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile to the corresponding acid with *E. coli* JM101 (pQE10-AtNIT1). Reaction was started with the addition of 9.3 mM substrate to 114 mL 0.1 M KPi buffer (pH 8.3) containing 50 g of cell dry weight/L. Four hours later an additional 15.3 mM substrate was added.

which is 3.5% of the activity of the free enzymes towards the same substrate and under the same conditions indicating that the protection of the active site did reduce the loss of activity somewhat. Given that the activity of the native enzyme for (**6**) is only 1.3% of the activity for 3-PPN an activity of perhaps 0.1 mU/mg immobilized protein might be expected for (**6**).

Third, we performed an experiment, in which (**6**) was hydrolyzed to (**7**) in vivo with *E. coli* JM101 (pQE10-AtNIT1) [24] carrying the genes of AtNIT1 from *A. thaliana* in shake-flasks. Almost 0.15 mM product was formed in 3 h (Fig. 7). Product formation was stable over the reaction time. Maximum specific activity was 0.85 U/g cdw and average specific activity was 0.5 U/g cdw, which is 0.2% of the activity obtained with the substrate 3-phenylpropionitrile under the same conditions.

To examine the stability of activity of whole cells at high cell densities in shake flasks over a longer period of time, we performed the biotransformations for 23 h with a cell density of 50 g cdw/L in shake flasks. Product concentration reached 11.7 mM (243 mg in 114 mL) at the end of the biotransformation (Fig. 8), equivalent to a yield of 48%. Maximum specific activity was 0.5 U/g cell dry weight, with a final activity at the end of the biotransformation of 0.17 U/g cell dry weight.

Among all three approaches for the hydrolysis of (**7**), immobilized AtNIT1 showed a very low activity, which restricts the practical applicability of immobilized AtNIT1. This might also be true for other multi-subunit nitrilases, and in fact we found no applications of immobilized nitrilases in the literature. A comparison of the process parameters of the other two approaches is summarized in Table 4. Incubation with isolated enzymes resulted in almost two times higher average specific activities and 1.6 times higher yield than incubation with whole cells. However, the recovery of the enzymes from the reaction mixtures for reuse can be a limitation for practical applications of isolated nitrilases. After the biotransformation only 43% of the isolated enzyme activity could be recovered.

Whole cell biocatalysts are easy to prepare for large-scale applications. Although the productivities of the whole cells are lower compared to productivities of isolated enzymes, high concentrations of cells can be used to increase the volumetric productivities.

An alternative approach to the synthesis of acidic chiral diols would be to clone the nitrilase genes together with the dioxygenase genes either on two compatible plasmids or on a single

Table 4
Process data for hydrolysis of *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile to the corresponding acid with AtNIT1 from *Arabidopsis thaliana*

Parameter	AtNIT1 from *Arabidopsis thaliana*			
	As isolated enzyme		In *E. coli* JM101 (pQE-10 AtNIT1)	
	Small scale	Large scale	Small scale	Large scale
Volume (mL)	1	55.5[a]	20	114
Protein amount (mg)	2.6	1332	17[b]	2850[b]
Total substrate amount (mg)	0.75	135	4.6	456
Reaction time (h)	2.5	10.6	3	23
Product amount (mg)	0.17	116	0.55	243
Yield (%)	20	77	11	48
Maximum specific activity (U/g protein)	2.3	4	1.7[b]	1.0[b]
Average specific activity (U/g protein)	2.3	0.74	1.0[b]	0.34[b]
Average vol. productivity (g product/L/day)	1.6	3.9	0.2	2.2

[a] Initial reaction volume. It increased to 66.6 mL with addition of substrate.
[b] Protein amount is estimated as 50% of the total cell dry weight.

plasmid for expression of the genes in the same host. This would enable multi-step biocatalysis in a single host and in a single reactor.

4. Conclusions

Chemoselective nitrilase hydrolysis of the *cis*-dihydrodiols with a nitrile group resulted in a new class of acidic chiral diols. Here we screened nitrilases for the hydrolysis of *cis*-1,2-dihydroxy-3-cyanocyclohexa-3,5-diene and *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile to the corresponding acids. A nitrilase from *Rhodococcus* sp. was found to hydrolyze both substrates while AtNIT1 from *A. thaliana* hydrolyzed only the latter. Isolated AtNIT1 in the form of free and immobilized enzyme, and recombinant *E. coli* carrying AtNIT1 were used for the hydrolysis of *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile. For practical applications, whole cell biocatalysis might be preferred for the hydrolysis of dihydrodiols with a nitrile group, since activity of the whole cells is higher and more stable than the activity of the immobilized enzymes. As well, preparation of the recombinant biocatalyst is straightforward and whole cells carrying AtNIT1 can be immobilized to increase the catalyst longevity in a hydrolysis process. Furthermore, combination of chlorobenzene dioxygenase and nitrilase in the same host could provide a new and efficient route to a previously undescribed class of acids and chiral *cis*-diols.

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