BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

From sequence to function: a new workflow for nitrilase identification

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Abstract

Nitrilases are industrially important biocatalysts due to their ability to degrade nitriles to carboxylic acids and ammonia. In this study, a workflow for simple and fast recovery of nitrilase candidates from metagenomes is presented. For identification of active enzymes, a NADH-coupled high-throughput assay was established. Purification of enzymes could be omitted as the assay is based on crude extract containing the expressed putative nitrilases. In addition, long incubation times were avoided by combining nitrile and NADH conversion in a single reaction. This allowed the direct measurement of nitrile degradation and provided not only insights into substrate spectrum and specificity but also in degradation efficiency. The novel assay was used for investigation of candidate nitrilase-encoding genes. Seventy putative nitrilase-encoding gene and the corresponding deduced protein sequences identified during sequence-based screens of metagenomes derived from nitrile-treated microbial communities were analyzed. Subsequently, the assay was applied to 13 selected candidate genes and proteins. Six of the generated corresponding Escherichia coli clones produced nitrilases that showed activity and one unusual nitrilase was purified and analyzed. The activity of the novel arylacetonitrilase Nit09 exhibited a broad pH range and a high long-term stability. The enzyme showed high activity for arylacetonitriles with a $K_M$ of 1.29 mM and a $V_{max}$ of 13.85 U/mg protein for phenylacetonitrile. In conclusion, we provided a setup for simple and rapid analysis of putative nitrilase-encoding genes from sequence to function. The suitability was demonstrated by identification, isolation, and characterization of the arylacetonitrilase.

Key points

• A simple and fast high-throughput nitrilase screening was developed.
• A set of putative nitrilases was successfully screened with the assay.
• A novel arylacetonitrilase was identified, purified, and characterized in detail.

Keywords Arylacetonitrilase • Metagenome • Nitrilase • Nitrilase assay • Phenylacetonitrile

Introduction

Nitriles are organic compounds that harbor $-C\equiv N$ as functional group. Many of these compounds are toxic. They are widespread in nature and present in plants as cyanoglycosides (Conn 1979), cyanohydrins in fungi and arthropods, different antibiotics in bacteria, or ricinine and phenylacetonitrile in plants (Jallageas et al. 1980).

For the enzymatic degradation of nitriles, two pathways are known (Supplementary Fig. S1). The first one is the direct conversion of nitriles to corresponding carboxylic acids and ammonia via nitrilases (EC 3.5.5.-). The second one involves the degradation of nitriles to corresponding amides via nitrile hydratases (NHases) (EC 4.2.1.84) and the subsequent hydrolysis of amides to carboxylic acids and ammonia using amidas (EC 3.5.1.4). Respective enzymes are present in bacteria (Egelkamp et al. 2017), filamentous fungi (Martíneková et al. 2009), yeasts (Rustler and Stolz 2007), and plants (Piotrowski 2008). The enzymes are used in industry for the production of...
bulk chemicals such as nicotinic acid or glycolic acid (Shaw et al. 2003; Panova et al. 2007). Novel nitrile-degrading enzymes, especially those with reduced substrate and/or product inhibition under production conditions, are of industrial relevance (Bui et al. 1984; Vaughan et al. 1989; Almatawah and Cowan 1999; Zhang et al. 2011a).

Many nitrile-degrading enzymes were found via (meta)genome mining techniques. A functional screening approach was applied to identify nitrilase-encoding genes from plasmid libraries containing metagenomic DNA from different sources (Robertson et al. 2004; Bayer et al. 2011; Soares Bragança et al. 2017). In addition, in silico screening of (meta)genomic sequence data was used to identify putative nitrilases and nitrile hydratases in publicly available databases (Vergne-Vaxelaire et al. 2013). Although sequence-based approaches lead very quickly to many new gene candidates for the targeted enzyme type, they remain as predictions until functional verification. To overcome this limitation and take advantage of the constantly growing metagenomic sequence pool, procedures are needed for efficient verification of nitrilase activity and identification of potential substrates of these enzymes.

The focus of this study was to establish a workflow that enables the fast functional verification of sequence-based, screening-derived putative nitrilases and their substrates. Six metagenomes, which were obtained during a study targeting the impact of different nitriles on microbial communities (Egelkamp et al. 2019), were chosen as starting material. A procedure was established to reduce the 70 putative nitrilase-encoding genes that had been inferred from sequence-driven mining of the metagenomes to candidates with verified enzyme activity. Serving as proof of concept, one enzyme candidate was heterologously expressed and purified. Subsequently, the recovered enzyme and its activity were characterized, including the determination of optimal pH, temperature range, stability, substrate specificity, and enzyme kinetics. In addition, influences of divalent ions and other substances on enzyme activity were determined.

**Material and methods**

**Origin of nitrilases**

Putative nitrilase-encoding genes were identified in, and amplified from, metagenomic DNA that originated from compost-derived enrichment cultures containing either phenylacetonitrile, succinonitrile, acetonitrile, crotononitrile, 4-hydroxybenzonitrile, or cyclohexanecarbonitrile (Egelkamp et al. 2019). The compost sample was collected in the Experimental Botanical Garden of Georg-August-Universität Göttingen (Germany; 51° 33’ 22.6” N, 9° 57’ 16.2” E). GenBank accession numbers of the annotated corresponding 6 metagenomes are as follows: phenylacetonitrile, RCUE00000000; succinonitrile, RCUP00000000; acetonitrile, RCUC00000000; crotononitrile, RCUN00000000; 4-hydroxybenzonitrile, RCUF00000000, and cyclohexanecarbonitrile, RCU00000000.

An already characterized aliphatic nitrilase from *Rhodococcus rhodochrous* K22 was used as positive control for establishing the high-throughput nitrilase assay (Kobayashi et al. 1990; Kobayashi et al. 1992b). For this purpose, the corresponding nitrilase gene was codon-optimized for *Escherichia coli* K12 derives using the web suite JCat (http://www.jcat.de) (Grote et al. 2005) and synthesized by Integrated DNA Technologies (Leuven, Belgium).

**Bioinformatic analysis of described and putative nitrilases**

Nitrilase sequence reference data were obtained from the SWISS-Prot database (Bairoch and Apweiler 2000; date of search: October 14, 2018). The data were further processed by removing misannotated enzymes, mere subunits, or nitrile hydratases designated as nitrilases. Clustering of data was done with CD-HIT (Huang et al. 2010). A fasta file containing the SWISS-Prot nitrilases and metagenome-deduced nitrilases was used as input and a sequence identity cutoff of 40% was set for clustering (Supplementary Data File S1).

**Growth medium**

Lysogeny broth (LB) (10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter) was used for growth of microorganisms. For solid media, 15 g agar per liter was added.

**Amplification of metagenome-encoded putative nitrilases**

PCR reaction mixture (total volume 50 μL) contained 10 μL 5-fold Phusion HF buffer, 200 μM of each dNTP, 0.2 μM of each primer (Supplementary Table S1), 3% DMSO, 50 ng metagenomic DNA as template and 1 U of Phusion polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Initial denaturation was performed at 98 °C for 5 min, followed by 30 cycles of denaturation at 98 °C for 30 s, annealing (temperature based on primer melting temperature) for 30 s and elongation at 72 °C for 45 s per kbp. The final elongation was for 5 min at 72 °C.

**Plasmids, strains, and transformation**

Amplified putative nitrilase-encoding genes (Supplementary Data File S2) were cloned into the pBAD18 vector system (Guzman et al. 1995). Chemically competent *E. coli* TOP10
cells were transformed according to the protocol of the manufacturer (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, cells were plated on 100 μg/mL ampicillin-containing LB plates and incubated overnight at 37 °C. The fidelity of the constructs was checked by Sanger sequencing (Microsynth Seqlab, Göttingen, Germany).

Heterologous expression of putative nitrilases and purification of His6-tagged proteins

E. coli TOP10 strains containing the pBAD18-based recombinant plasmids were grown in LB medium at 180 rpm (Innova 44 shaker, New Brunswick Scientific, Nüteringen, Germany) and 37 °C overnight. The preculture was used to inoculate 10 mL LB medium with an OD600 of 0.1. Subsequently, the culture was incubated for 1.5 h at 37 °C and 180 rpm (Innova 44 shaker) to a final OD600 of 0.6–0.8. For induction of heterologous gene expression, 1.5% L-(+) arabinose was added, followed by 6 h of incubation.

Cells containing the produced (His6-tagged) nitrilase were washed twice with 1× LEW buffer of the Protino Ni-TED kit (Macherey-Nagel, Düren, Germany) and resuspended in 1.5 mL of the same buffer containing 40 μg/mL DNase I and 0.1% (w/v) lysozyme. The cells were then disrupted by at least three passages through a French press at 1.38 × 10^8 Pa (Thermo Fisher Scientific). The extract was cleared by centrifugation at 6000×g and 4 °C for 20 min. The recovered supernatant was loaded onto Protino Ni-TED columns according to the protocol of the manufacturer (Macherey-Nagel). The purified enzyme was further analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Protein concentration was determined using the Bradford method (Bradford 1976) with bovine serum albumin as standard.

Substrates for nitrilase screenings

Stock solutions of nitriles (final concentration of 2.7 M) were generated by solving the nitriles in N,N-dimethylformamide (DMF) (Merck KGaA, Darmstadt, Germany). These stock solutions were sterile-filtered and stored at 4 °C. The following nitriles were used: phenylacetonitrile, acetonitrile (both TCI Deutschland GmbH, Eschborn, Germany), succinonitrile, crotononitrile, 4-hydroxybenzonitrile, acetone cyanohydrin, cyclohexanecarbonitrile, fumaronitrile, and 2-phosphopropionitrile (all Sigma-Aldrich Chemie GmbH, Munich, Germany). In addition, mandelonitrile, 2-phenylbutyronitrile, benzonitrile, 3-indoleacetonitrile, 2-thiophenecarbonitrile, 3-thiopheneacetonitrile, (2-chlorophenyl)acetonitrile, (3-chlorophenyl)acetonitrile, (4-chlorophenyl)acetonitrile, cinnamonic acid, 1,4-phenylenediacetonitrile, 2-naphthylacetonitrile (all Sigma-Aldrich), and 3-phenylpropionitrile (Alfa Aesar, Haverhill, MA, USA) were used for further characterization of the purified ary lacetonitrilase.

High-throughput nitrilase activity assay

Degradation of nitriles was measured by monitoring the release of ammonia in a coupled enzymatic reaction (Reisinger et al. 2006). The ammonia reacts with NADH and α-ketoglutarate in the presence of a glutamate dehydrogenase from bovine liver type II (GDH; Sigma-Aldrich) to glutamate. The reduced co-factor is monitored by its absorbance change at 340 nm. For screening of putative nitrilases, the high-throughput assay of Vergne-Vaxelaire (Vergne-Vaxelaire et al. 2013) was adjusted to allow real-time measurements without time-consuming incubation steps. The assay was performed in a flat-based 96-well plate. The reaction mixture (final volume 250 μL) contained 0.5 mM NADH, 1 mM α-ketoglutarate, 37 μL of a 2.7-M nitrile stock solution (solved in DMF), 20 μg crude extract (added at the end), 1 U/mL GDH, and 50 mM Tris-HCl (pH 8.0) buffer. Crude extract containing the pBAD18 crude extract vector without insert was used as negative control. The reaction mixture was incubated at 37 °C in a Synergy 2 microplate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany). The absorbance was constantly measured for 1.5 h at 340 nm. For calculation of enzyme activity, the values of the pBAD18 crude extract negative control were subtracted from that of the crude extract containing putative nitrilases. The activity of the nitrilase from R. rhodochrous K22 (positive control) with different substrates was described previously (Kobayashi et al. 1990; Kobayashi et al. 1992b). During the first 15 min of measurement using the control enzyme, the degradation of succinonitrile, fumaronitrile, and crotononitrile was observed and the absorbance decrease was at least 0.75. Thus, only reactions completed during the first 15 min of measurement and showing at least an absorbance decrease of 0.75 were considered as nitrilase activity and selected for the subsequent analyses.

Construction of a phylogenetic tree

For calculation of a phylogenetic tree, MEGA X version 10.1.7 (Kumar et al. 2018) was used. Fifteen characterized nitrilases and 3 amidases were recovered from the NCBI database and used as references. Arylacetonitrilases were derived from Alcaligenes faecalis JM3 (D13419; Kobayashi et al. 1993), Pseudomonas putida MTCC 5110 (EF467660; Banerjee et al. 2009), and Pseudomonas fluorescens EBC191 (AY885240; Kiziak et al. 2005). For aliphatic nitrilases, the enzymes from Rhodococcus rhodochrous K22 (D12583; Kobayashi et al. 1992b), Comamonas testosteroni (L32589; Lévy-Schil et al. 1995), and Synechocystis sp. PCC6803 (BA000022; Heinemann et al. 2003) were used as references. Sequences for aromatic nitrilases were obtained.
from *Rhodococcus rhodochrous* J1 (D11425; Kobayashi et al. 1992a), *R. rhodochrous* NCIMB 11216 (AX235749; Ress-Löschke et al. 2001), and *Aeribacillus pallidus* RAPc8 (DQ826045; Williamson et al. 2010). References for cyanide dihydrotases were taken from *Pseudomonas stutzeri* AK61 (KM459551; Crum et al. 2015), *Bacillus pumilus* C1 (AF492815; Jandhyala et al. 2003), and *B. pumilus* 8A3 (AF492814; Jandhyala et al. 2003). β-Cyano-β-alanine nitrilases originated from *Pseudomonas protegens* Pf-5 (CP000076; Paulsen et al. 2005; Howden et al. 2009), *P. fluorescens* Pf0-1 (CP000094; Howden et al. 2009; Silby et al. 2009), and *Pseudomonas pseudoalcaligenes* CECT 5344 (HG916826; Wibberg et al. 2014; Acera et al. 2017). In addition, amidases from *R. rhodochrous* M8 (AY971668; Riabchenko et al. 2006), *P. aeruginosa* PAO1 (AE004091; Ambler et al. 1987), and *P. fluorescens* SBW25 (AM181176; Howden et al. 2009; Silby et al. 2009) were included. The reference sequences and 6 putative nitrilases identified in this study were aligned with MUSCLE (Edgar 2004), leading to 1401 positions in the final dataset. Subsequently, evolutionary analysis was performed using the Maximum Likelihood method with the General Time Reversible model (Nei and Kumar 2000) and a “very weak” branch swap filter. Evolutionary rate differences among sites were modeled with a discrete Gamma distribution (5 categories; +G, parameter = 2.0817). The tree with the highest log likelihood (−20,729.17) is shown. Test of phylogeny was performed by calculation of 1000 bootstrap replicates, and nodes with values below 50% were condensed for the final image.

### Nitrilase characterization

The activity of a novel arylacetonitrilase (Nit09) was characterized by using a colorimetric assay for detection of ammonia (Fawcett and Scott 1960). As the reagents are temperature-sensitive, all experiments were performed in an air-conditioned laboratory at 23 °C. Nitrilase reaction was performed in a 500-μL mixture containing 25 μL nitrile (100 mM solved in DMF), 1.25 μg purified His6-tagged nitrilase, and 0.1 M citrate-phosphate buffer (pH 6.0) for 2 min at the temperature optimum of 50 °C. Then, 111 μL of this reaction was transferred into 222 μL sodium phenoxide (0.266 M phenol and 4 M NaOH), followed by 333 μL of 0.01% sodium nitroprusside and 333 μL of 0.02 N hypochlorite. Ammonia reacts with sodium phenoxide and hypochlorite to produce indophenol blue. Sodium nitroprusside improves the intensity, reproducibility, and stability of the blue color (Kaplan et al. 1965). The reaction mixture was incubated for 15 min at 27 °C in darkness. Subsequently, absorption was measured at 630 nm. To determine the substrate spectrum of Nit09, 20 mM of the tested nitrile was used and the reaction mixture was incubated for 15 min.

### Nitrilase inhibitors and enzyme stability

Effects of different potential inhibitors (EDTA, DTT, H2O2, HgCl2, AgNO3, CaCl2, MnSO4, MgSO4, FeSO4, CuSO4, CoSO4, ZnCl2, NaN3, and SDS) and solvents (methanol, ethanol, glycerol, isopropanol, DMSO, acetone, chloroform, and toluene) on Nit09 activity were tested. Incubation was carried out at 50 °C for 2 min in the standard reaction mixture containing a putative inhibitor at 1 mM or 5% (v/v) and 20% (v/v) of tested organic solvent. For stability analysis, the His6-tagged purified Nit09 was stored in Protino® Ni-TED elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 8.0) containing 1 mM NaN3 at 4 °C in the dark for 91 days. Enzymatic activity was determined under standard reaction conditions.

### Determination of enzymatic constants

The steady-state kinetics for Nit09 activity was measured in the presence of GDH to detect the concomitant oxidation of NADH at 340 nm and 37 °C using a UV/Vis spectrophotometer (Cary 100 UV-Vis, Varian Medical Systems, Darmstadt, Germany). The reaction mixture (final volume 1 mL) contained 0.5 mM NADH, 1 mM α-ketoglutarate, 0.1 mM to 100 mM of phenylacetonitrile (final volume 37 μL), 5 μg purified nitrilase Nit09, 1 U/mL GDH from bovine liver type II (Sigma-Aldrich), and 0.1 M HEPES buffer (pH 8.0). Negative controls were prepared without nitrilase. To determine the turnover number and the maximum number of chemical conversions of phenylacetonitrile molecules per second that a single catalytic site will execute for a given enzyme concentration, the slope (ΔA340) had to be first determined. This was done by the software of Cary WinUV version 3.0 (Varian Medical Systems). With the slope, the kcat values were determined and plotted against phenylacetonitrile concentrations. Further, the constants kcat and Km were analyzed using the Michaelis-Menten equation of the R package “drc” (Ritz et al. 2015).

### Results

#### Optimization of the NADH-coupled nitrilase assay

Most current high-throughput nitrilase assays have drawbacks such as low sensitivity (Yazbeck et al. 2006), the requirement of specific hardware like fluorometers (Banerjee et al. 2003), or suitability only for specific nitriles (Zhu et al. 2007b). A promising approach is a NADH-coupled assay in which ammonia released by the nitrilase reaction is consumed by a glutamate dehydrogenase (GDH) in the course of the conversion of NADH to NAD. The conversion of this cofactor is monitored at 340 nm and reveals activity of the tested (putative) nitrilases (Vergne-Vaxelaire et al. 2013). However,
it requires extended incubation times for nitrilase reaction and provides only qualitative information and no insights into enzymatic efficiency. We modified the assay to allow fast and simple screenings of nitrilase activity with different substrates without additional incubation steps in a single reaction mixture. Therefore, concentrations of all test ingredients were optimized to facilitate continuous measurement of NADH conversion in a 96-well microtiter plate providing first insights into the efficiency of nitrile degradation. In comparison to the method of Vergne-Vaxelaire (Vergne-Vaxelaire et al. 2013), the amount of NADH was increased to 0.5 mM for a larger measurement range and the concentration of α-ketoglutarate was reduced to prevent inhibition of the GDH reaction. These modifications were tested using crude extract containing an already characterized nitrilase from Rhodococcus rhodochrous K22 (Kobayashi et al. 1990; Kobayashi et al. 1992b) as proof of principle (Fig. 1).

To minimize the identification of false positives or enzymes with low activity, two thresholds were defined. During the first 15 min of measurement, total conversion of succinonitrile, fumaronitrile, and crotononitrile by the K22 control nitrilase was recorded and the absorbance decrease at 340 nm was at least 0.75. As K22 nitrilase activity for these substrates was described previously (Kobayashi et al. 1990; Kobayashi et al. 1992b), only reactions active in the first 15 min and with an absorbance decrease of at least 0.75 were considered as positive for substrate conversion and nitrilase activity during subsequent experiments.

Screening of putative metagenome-derived nitrilase-encoding genes and selection of candidates for activity analysis of the corresponding enzymes

Six metagenomes of microbial communities individually obtained from phenylacetonitrile-, succinonitrile-, acetonitrile-, crotononitrile-, 4-hydroxybenzonitrile-, or cyclohexanecarbonitrile-containing enrichment cultures (Egelkamp et al. 2019) served as sources for putative nitrilases. In total, 70 annotated putative nitrilase-encoding genes were identified in these metagenomes (Supplementary Data File S2). In a first step, the sequences of the identified genes and the corresponding deduced proteins were analyzed (Supplementary Data File S2). Initially, to relate the deduced protein sequences with already characterized enzymes, all deduced protein sequences of the metagenome-derived nitrilase candidates were compared with the amino acid sequences of 46 characterized reference nitrilases obtained from SWISS-Prot. This analysis resulted in 13 nitrilase protein clusters (Supplementary Data File S3) and reduced our data set to 60 unique candidates. The first three clusters contained characterized nitrilases obtained from SWISS-Prot as well as our putative metagenome-derived nitrilases with molecular masses ranging from 5.96 to 38.68 kDa (cluster 1), 12.7 to 40.2 kDa (cluster 2), and 10.41 to 38.62 kDa (cluster 3). Clusters 4, 6, 10, 11, 12, and 13 consisted solely of characterized SWISS-Prot-derived nitrilases, whereas the remaining four clusters (5, 7, 8 and 9) included only our putative metagenome-derived enzyme sequences. As the smallest characterized prokaryotic nitrilase originates from Pyrococcus abyssi GE5 and consists of 262 amino acids (Mueller et al. 2006), all putative nitrilases smaller than this were checked for the presence of ribosomal binding sites (RBS) in the corresponding metagenomic DNA region to exclude artificial peptides caused by incomplete metagenomic assembly or misassembly. Finally, 37 candidates were selected for cloning and overexpression in E. coli.

After amplification of the respective genes from metagenomic DNA and sequence verification, frameshift mutations were observed in four cases, leading to their exclusion. In 31 cases, variations resembling point mutations of the metagenome-derived gene sequences were recorded. Probably, the sequencing depth of the metagenomes was not sufficient to distinguish between very similar sequences, leading to the amplification of previously unidentified gene variants. All of these variations were included in subsequent analyses. In total, 26 putative nitrilase-encoding genes were successfully subcloned (Supplementary Data File S2). Analysis of heterologous protein formation revealed production of 13 putative nitrilases by the E. coli host. Although the remaining 13 putative nitrilases could be successfully overexpressed, the corresponding enzymes were not present in the cleared cell-

Fig. 1 Nitrile degradation by the Rhodococcus rhodochrous K22 nitrilase. The assay was performed in triplicate at 37 °C in a 96-well microtiter plate. NADH conversion was monitored at 340 nm. Fumaronitrile, succinonitrile, and crotononitrile were degraded after 15 min, thereby defining the threshold (red line) for the required experimental time...
free crude extract. These enzymes were detected in the pellet that remains after cell disruption and clearing the crude extract by centrifugation. These enzymes were not considered for subsequent analysis, as we assumed that these proteins were part of inclusion bodies.

The previously established assay was used to verify nitrilase activity with the 13 putative nitrilase candidates using nine different nitriles as substrate (Table 1). Six of the putative enzymes showed nitrilase activity with at least one nitrile. Most were active with succinonitrile or fumaronitrile. In addition, one of these (Nit09) could act on the aromatic phenylacetonitrile (Fig. 2).

Gene sequences of the six active enzymes and already characterized reference nitrilases were used to calculate a phylogenetic tree (Fig. 3). The phenylacetonitrile-specific nitrilase (Nit09) clustered with arylacetonitrilases, whereas the four

Table 1 Origin and substrate specificity of expressed putative nitrilases

| Nitrilase | Metagenomic origin<sup>a</sup> | Size (bp)/mass (kDa) | Closest relative<sup>b</sup> | Substrate specificity<sup>c</sup> | NCBI accession number |
|-----------|-------------------------------|---------------------|-----------------------------|-------------------------------|----------------------|
| Nit09     | SUN                           | 1005/36.12          | *Variovorax boronicumulans* J1 nitrilase (Q: 100%, E: 0.0, I: 98.1%, A: KY937903) | PAN               | MN689843             |
| Nit10     | SUN                           | 924/33.00           | *Pseudomonas fluorescens* NCIMB 11764 nitrilase (Q: 99%, E: 0.0, I: 87.7%, A: CP010945) | –                 | MN689844             |
| Nit14     | SUN                           | 975/35.13           | *Pseudomonas* sp. S34 aliphatic nitrilase (Q: 100%, E: 0.0, I: 96.3%, A: CP019398) | SUN, FUN          | MN689845             |
| Nit24     | ACN                           | 1068/38.70          | *Janthinobacterium* sp. Marseille nitrilase (Q: 99%, E: 0.0, I: 86.9%, A: CP000269) | –                 | MN689846             |
| Nit28     | ACN                           | 1059/38.62          | *Pseudomonas* sp. LAB-08 nitrilase (Q: 97%, E: 0.0, I: 88.9%, A: AP017423) | SUN, FUN          | MN689847             |
| Nit30     | ACN                           | 924/32.93           | *Pseudomonas fluorescens* NCIMB 11764 nitrilase (Q: 99%, E: 0.0, I: 87.4%, A: CP010945) | –                 | MN689848             |
| Nit37     | ACN                           | 1017/36.74          | *Cupriavidus basilensis* 4G11 nitrilase (Q: 98%, E: 0.0, I: 86.7%, A: CP010537) | SUN, FUN          | MN689849             |
| Nit56     | CRN                           | 975/35.20           | *Pseudomonas* sp. UW4 nitrilase (Q: 100%, E: 0.0, I: 97.4%, A: CP003880) | SUN, FUN          | MN689850             |
| Nit57     | CRN                           | 975/35.18           | *Pseudomonas* sp. UW4 nitrilase (Q: 100%, E: 0.0, I: 98.0%, A: CP003880) | –                 | MN689851             |
| Nit59     | CRN                           | 924/32.84           | *Pseudomonas* sp. UW4 nitrilase (Q: 100%, E: 0.0, I: 95.1%, A: CP003880) | –                 | MN689852             |
| Nit60     | CRN                           | 924/32.86           | *Pseudomonas* sp. S34 nitrilase (Q: 100%, E: 0.0, I: 98.6%, A: CP019398) | FUN               | MN689853             |
| Nit66     | CRN                           | 924/32.91           | *Pseudomonas* sp. UW4 nitrilase (Q: 100%, E: 0.0, I: 97.4%, A: CP003880) | –                 | MN689854             |
| Nit78     | CCN                           | 924/32.83           | *Pseudomonas* sp. S34 nitrilase (Q: 100%, E: 0.0, I: 99.2%, A: CP019398) | –                 | MN689855             |

<sup>a</sup> Metagenomic origin of putative nitrilase genes. SUN, succinonitrile treatment; ACN, acetonitrile treatment; CRN, crotononitrile treatment; CCN, cyclohexanecarbonitrile treatment

<sup>b</sup> Gene sequences were used for a blastn search against the NCBI nr database and best hits are shown. Q, query cover; E, e value; I, identity; A, accession

<sup>c</sup> Substrate specificity determined with high-throughput assay. Tested substrates were PAN, phenylacetonitrile; SUN, succinonitrile; ACN, acetonitrile; CRN, crotononitrile; HBN, 4-hydroxybenzonitrile; ACH, acetone cyanohydrin; CCN, cyclohexanecarbonitrile; FUN, fumaronitrile; PPN, 2-phenylpropionitrile; –, no enzymatic activity with tested substrates
nitrilases active with succinonitrile and fumaronitrile (Nit14, Nit28, Nit37, and Nit56) form a branch with an aliphatic nitrilase from *Synechocystis* sp. PCC6803, and the fumaronitrile-degrading nitrilase (Nit60) most closely resembled with three β-cyano-L-alanine nitrilases.

**Characterization of arylacetonitrilase Nit09**

Phenylacetonitrile belongs to the class of arylacetonitriles, a subclass of nitriles degraded by the rare group of arylacetonitrilases (EC 3.5.5.5). We chose the phenylacetonitril-degrading enzyme candidate Nit09 for detailed characterization and verification of enzymatic activity under defined conditions as it represented probably a novel arylacetonitrilase. This assumption was further supported by the clustering of nit09 with other arylacetonitrilases in the previously calculated phylogenetic tree (Fig. 3). The His6-tagged enzyme was produced and purified by immobilized metal ion affinity chromatography. SDS-PAGE analysis of the enzyme preparation confirmed production of a 36 kDa enzyme, which is in accordance with the Nit09 molecular mass deduced from the gene sequence (data not shown). Phenylacetonitrilase was used as substrate to further characterize the purified enzyme, since Nit09 showed the ability to degrade phenylacetonitrile in the high-throughput screening.

Enzyme activity was highest at pH 6 with approximately 90% activity being retained between pH 5.5 and 8. At both pH 5 and pH 10.5, the relative activity dropped to 20% (Fig. 4a). Optimum temperature of enzyme activity was at 50 °C, whereas nitrilase activity was reduced to 30% at 60 °C (Fig. 4b). Furthermore, stability of the nitrilase was tested over several weeks under storage conditions at 4 °C. Remaining catalytic activity of > 80% was observed during the first 3 weeks, followed by a slow decline (Fig. 5). After 3 months, 8% of the initial activity remained.

To further examine the substrate spectrum, the Nit09 nitrilase was initially tested with the same substrates used for the high-throughput assay. In this way, the results of the above-described screening assay were verified and phenylacetonitrilase was the only one of these substrates converted by Nit09 under the tested conditions (Table 2). To determine activity of the enzyme with structurally related compounds, 13 aromatic nitriles were additionally tested. With four of them (2-phenylbutyronitrile, benzonitrile, 2-naphthylacetonitrile, and cinnamonitrile), nitrilase activity was not detected. With 2-chlorophenylacetonitrile, 3-chlorophenylacetonitrile, and 4-chlorophenylacetonitrile as substrates, only low relative activities (5 to 12%) were recorded compared to phenylacetonitrile. Reduced relative activity

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**Fig. 2** Substrate specificity of Nit09. The assay was performed at 37 °C in a 96-well plate. NADH conversion was monitored at 340 nm. Degradation of phenylacetonitrile was followed until the previously determined 15 min threshold (red line)

**Fig. 3** Phylogenetic nitrilase tree. Metagenomic sequences used in this study are in bold letters. PAN phenylacetonitril, SUN succinonitril, FUN fumaronitril, ARYL arylacetonitril, ALIPH aliphatic nitrilase, ARO aromatic nitrilase, CN cyanide dihydratase, Ala(CN) β-cyano-L-alanine nitrilase

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was also detected for 3-phenylpropionitrile (16%), 1,4-phenylene diacet onitrile (19%), mandelonitrile (22%), and 3-indoleacetonitrile (34%). Strikingly, significantly higher relative activity was detected for 3-thiopheneacetonitrile (188%) and 2-thiopheneacetonitril (385%).

To assess the effect of potential inhibitors and solvents on enzyme activity, the activity of the purified enzyme was tested in the presence of different chemicals. For detection of thiol residues essential for enzyme activity, the thiol-binding reagents HgCl₂ and AgNO₃ were applied to the reaction mixture. The recorded decline of enzyme activity to 1.6 and 2.0%, respectively, indicated that the functionality of Nit09 depends on these residues (Table 3). As the metal-chelating agent EDTA had no significant effect on enzymatic activity, dependence on divalent metal cofactors is not indicated. This was further supported by the lack of any effect when divalent ions such as Mg²⁺, Fe²⁺, and Mn²⁺ were added to the reaction mixtures. Only addition of Zn²⁺ resulted in a slight relative activity increase to 114%.

Strong inhibitory effects were encountered in the presence of DMSO, acetone, and chloroform even at 5% concentration (Table 4). At this concentration, the nitrilase was less sensitive to methanol, ethanol, isopropanol, and glycerol treatment. At 20% concentration, only glycerol did not abolish enzymatic activity.

Finally, kinetic constants of Nit09 were determined. The reaction rate increased with phenylacetonitrile concentration until a saturation was reached at 6 mM. Between 10 and 50 mM, a rapid decrease of activity was recorded, followed by a slow decline up to 100 mM (Fig. 6a), indicating inhibition by the substrate. Based on these data, kinetic constants were calculated with a non-linear model due to superior precision compared to linear methods like Lineweaver-Burk plots (Cho and Lim 2018). Phenylacetonitrile was converted at 37 °C with a $K_M$ of 1.29 mM and $V_{max}$ of 13.85 U/mg, reaching the saturation point at 6 mM (Fig. 6b).

## Discussion

### From putative nitrilase sequences to nitrilase function

In this study, a workflow and a fast and simple assay for screening of functional nitrilases was established. In comparison to the previously described method (Vergne-Vaxelaire et al. 2013), it reduces the total screening time from approx. 5 h to approximately 45 min by combining nitrilase reaction and subsequent ammonia conversion in a single reaction. The use of crude extract is less time-consuming compared to methods demanding purified enzymes. Furthermore, the assay system allows continuous measurement of nitrile degradation and provides insights into the efficiency and substrate spectra of the tested enzymes. However, its precision is limited by the GDH as only nitrilase reactions slower than that of GDH can be monitored in detail. In addition, other enzymes in the crude extract might cause a NAD release or regeneration of NAD to NADH, thereby influencing the measurement. To minimize the effect of these factors, additional precautions were taken. Most important is the use of crude extract containing the expression vector without insert as negative control. To further reduce the likelihood of false positives, a time limit for nitrilase reaction was used. Based on the control reactions observed with the well-characterized *R. rhodochrous* K22 nitrilase (Kobayashi et al. 1990; Kobayashi et al. 1992b), these thresholds were set to 15 min and an absorbance decrease at 340 nm of at least 0.75.

Many of the 70 putative metagenome-derived nitrilase candidate genes were not considered for functional verification. Reason for exclusion was redundancy or problems during expression. In the end, 13 putative nitrilases were screened against 9 different nitriles with 6 of them showing activity for at least one of the nitriles. As nitrilases are known to cluster based on their substrate spectrum (Howden and Preston 2009), the observed substrate specificity was further
controlled by calculation of a phylogenetic tree consisting of the 6 active enzymes and characterized nitrilases. The specificity of Nit09 was underlined by its clustering with arylacetonitrilases, as its substrate phenylacetonitrile converted in the initial screening belongs to this class of chemicals. In contrast, the fumaronitrile-specific nitrilase Nit60 contributed to a branch with $\beta$-cyano-L-alanine nitrilases. To our knowledge, the three $\beta$-cyano-L-alanine nitrilases have not been tested with fumaronitrile or other dinitriles (Howden et al. 2009; Acera et al. 2017), while our test substrates did not contain $\beta$-cyano-L-alanine. Thus, we can only assume that Nit60 would show activity with this compound and that the $\beta$-cyano-L-alanine nitrilases harbor a so far unknown ability to degrade dinitriles. Furthermore, a cluster consisting of four succinonitrile- and fumaronitrile-degrading nitrilases and a characterized nitrilase from *Synechocystis* sp. PCC6803 was identified. While the PCC6803-derived nitrilase barely acts on mononitriles, it is highly active for fumaronitrile (Heinemann et al. 2003), further supporting the results of the high-throughput assay. We could show that a combination of targeted sequence data analyses with a high-throughput activity assay assists in the fast identification of functional nitrilase candidates. Another possible application of the high-throughput activity assay is to employ it for the rapid substrate spectrum determination of known or recovered active nitrilases with a variety of different nitriles. The subsequent isolation and verification of the phenylacetonitrile-degrading nitrilase finally proved the reliability of the novel nitrilase screening workflow, which provides a way to successfully

### Table 2

| Substrate                      | Relative activity (%) a |
|--------------------------------|------------------------|
| Phenylacetonitrile             | 100.00 ± 1.19          |
| Succinonitrile                 | 0.17 ± 0.03            |
| Acetonitrile                   | 0.00 ± 0.01            |
| Crotononitrile                 | 0.08 ± 0.01            |
| 4-Hydroxybenzonitrile          | 0.00 ± 0.00            |
| Acetone cyanohydrin            | 0.13 ± 0.02            |
| Cyclohexanecarbonitrile        | 0.09 ± 0.01            |
| Fumaronitrile                  | 0.45 ± 0.04            |
| 2-Phenylpropionitrile          | 0.52 ± 0.05            |
| Mandelonitrile                 | 22.01 ± 0.61           |
| 2-Phenylbutyronitrile          | 0.07 ± 0.01            |
| 3-Phenylpropionitrile          | 15.81 ± 0.80           |
| Benzonitrile                   | 0.48 ± 0.32            |
| 2-Naphthylacetonitrile         | 0.11 ± 0.00            |
| 1,4-Phenylenediacetonitrile    | 18.82 ± 0.62           |
| 2-Chlorophenylacetonitrile     | 11.69 ± 0.80           |
| 3-Chlorophenylacetonitrile     | 5.34 ± 0.06            |
| 4-Chlorophenylacetonitrile     | 4.95 ± 0.17            |
| Cinnamonicitrile               | 0.12 ± 0.01            |
| 3-Indoleacetonitrile           | 34.38 ± 0.78           |
| 2 Thiophenecetonitrile         | 385.46 ± 0.78          |
| 3 Thiopheneacetonitrile        | 187.53 ± 5.75          |

a Activity with phenylacetonitrile as substrate (5.19 U/mg) was set as 100% relative activity

### Table 3

| Compound                  | Relative activity (%)a |
|---------------------------|------------------------|
| Without additives         | 100.00 ± 0.64          |
| EDTA                      | 104.45 ± 1.62          |
| DTT                       | 76.21 ± 3.29           |
| H$_2$O$_2$                | 24.56 ± 2.35           |
| HgCl$_2$                  | 1.57 ± 0.36            |
| AgNO$_3$                  | 1.96 ± 0.18            |
| CaCl$_2$                  | 106.29 ± 2.71          |
| MnSO$_4$                  | 98.01 ± 3.80           |
| MgSO$_4$                  | 101.87 ± 5.92          |
| FeSO$_4$                  | 98.25 ± 5.65           |
| CuSO$_4$                  | 86.98 ± 3.82           |
| CoSO$_4$                  | 81.01 ± 2.60           |
| ZnCl$_2$                  | 114.00 ± 2.04          |
| Sodium azide              | 98.89 ± 2.13           |
| SDS                       | 12.02 ± 2.77           |

a Activity with phenylacetonitrile as substrate without additional additives (5.68 U/mg) was set as 100% relative activity
narrow down bioinformatically identified enzyme candidates to the interesting functional representatives.

### A novel and stable arylacetonitrilase

Arylacetonitrilases (EC 3.5.5.5) are a rare group of enzymes, and to our knowledge, only 12 bacterial nitrilases of this type have been characterized (Table 5). Thus, the phenylacetonitrile-degrading enzyme Nit09 was chosen for further validation of the workflow, as its characterization also contributes to the knowledge on arylacetonitrilases. According to sequence similarity, nit09 is affiliated to *Variovorax boronicumulans* (Table 1). Nitrile-degrading abilities of this species have been described previously, but phenylacetonitrile degradation has not been mentioned (Nielsen et al. 2007; Zhang et al. 2012; Egelkamp et al. 2017; Sun et al. 2018). The molecular mass of Nit09 (36 kDa) is close to molecular masses of other known arylacetonitrilases such as the enzymes from *Pseudomonas* sp. UW4 (33 kDa; Duca et al. 2014), *Bradyrhizobium japonicum* USDA110 (37 kDa; Zhu et al. 2007a), or *P. fluorescens* EBC191 (38 kDa; Kiziak et al. 2005). In contrast, the broad pH range of Nit09 activity is not that common for nitrilases of this type, including the enzymes from *Alcaligenes faecalis* JM3 (Nagasawa et al. 1990) and *A. faecalis* ATCC 8750 (Yamamoto et al. 1992). In addition, Nit09 exhibits an unusual long-term stability with 80% remaining activity after 3 weeks of incubation at 4 °C; in contrast, the arylacetonitrilases from *A. faecalis* JM3 and *P. fluorescens* EBC191 lose up to 80% activity in 10 days.

During initial screening, minor degradation of other nitriles was observed in the crude extract containing Nit09. None of these substrates was degraded by the purified enzyme, underlining the importance of the 15-min threshold to avoid identification of false positive candidates.

Additional tests with substrates structurally related to phenylacetonitrile provided a deeper insight into specificity of the nitrilase. Weak activity on non-arylacetonitriles was only recorded for 3-phenypropionitrile, demonstrating the importance of an acetonitrile-like residue attached to the aryl structure for catalytic function. Halogenic ortho, meta, and para substitutions at the benzyl residue led compared with phenylacetonitrile as substrate to a strong reduction of enzymatic activity. This behavior is not common for other arylacetonitrilases such as the enzymes from *A. faecalis* JM3 (Nagasawa et al. 1990), *A. faecalis* ATCC 8750 (Yamamoto et al. 1992), and *P. putida* MTCC 5110 (Banerjee et al. 2006), as in all tested cases at least one type of substitution led to

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**Table 4** Effect of different organic solvents on the activity of Nit09. Reactions were run for 2 min at 50 °C using 0.1 M citrate-phosphate buffer (pH 6.0), 1.25 μg purified protein, and 5 mM phenylacetonitrile.

| Solvent     | Relative activity with 5% v/v organic solvent (%) | Relative activity of enzyme with 20% v/v organic solvent (%) |
|-------------|---------------------------------------------------|-------------------------------------------------------------|
| Without     | 100.00 ± 7.49                                     | 100.00 ± 7.49                                               |
| Methanol    | 88.01 ± 6.18                                      | 9.26 ± 1.97                                                 |
| Ethanol     | 85.22 ± 7.12                                      | 4.82 ± 0.41                                                 |
| Glycerol    | 81.11 ± 8.68                                      | 76.65 ± 9.06                                                |
| Isopropanol | 77.55 ± 9.43                                      | 3.63 ± 0.91                                                 |
| DMSO        | 4.80 ± 1.06                                       | 2.32 ± 0.72                                                 |
| Acetone     | 28.96 ± 4.79                                      | 0.14 ± 0.07                                                 |
| Chloroform  | 24.81 ± 3.44                                      | 3.70 ± 1.38                                                 |
| Toluene     | 8.12 ± 2.13                                       | 2.44 ± 1.60                                                 |

*a Activity with phenylacetonitrile as substrate without additional additives (5.68 U/mg) was set as 100% relative activity.

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**Fig. 6** Influence of substrate concentrations on the reaction rate of Nit09. Reactions were performed with phenylacetonitrile as substrate under standard conditions at 37 °C. a Steady-state measurement until 100 mM substrate concentration. b Steady-state measurement until saturation point at 6 mM substrate concentration.
increased activity. Interestingly, 1,4-phenylenediacetonitrile was converted with 19% relative activity, indicating an interference of halogens with the active center. Reduced activity was also observed for the plant hormone predecessor 3-indoleacetonitrile. The larger aromatic structure seems to cause general steric problems, as this is also the case for other arylacetonitrilases such as the enzymes from *A. faecalis* JM3 (Nagasawa et al. 1990), *Burkholderia cenocepa*cia J2315 (Wang et al. 2013), *P. fluorescens* EBC191 (Kiziak et al. 2005), and *P. putida* MTCC 5110 (Banerjee et al. 2006) exhibited reduced activity with this compound. In contrast, the heteroaromatic structure found in thiophene results in increased enzymatic activity compared to phenylacetonitrile, probably due to the assistance of the sulfur in the nucleophilic attack performed by nitrilases (Ramteke et al. 2013). Furthermore, an effect of the position of the heteroatom was recorded. 2-Thiopheneacetonitrile was converted more than twice as efficient as 3-thiopheneacetonitrile by Nit09. This strong influence of the heteroatom is typical for arylacetonitrilases and can lead to more than a tenfold difference in enzymatic activity (Nagasawa et al. 1990; Yamamoto et al. 1992; Kiziak et al. 2005).

Nit09 was strongly inhibited by the thiol-specific reagents AgNO$_3$ and HgCl$_2$. Most likely, thiol groups build an important part of the active site, which is in line with the proposed Cys-Glu-Lys catalytic triad of nitrilases (Fernandes et al. 2006). Most arylacetonitrilases such as the enzymes from *A. faecalis* JM3 (Nagasawa et al. 1990), *A. faecalis* ATCC 8750 (Yamamoto et al. 1992), and *A. faecalis* ZJUTB10 (Liu et al. 2011) are also inhibited by other heavy metal ions like Cu$^{2+}$ or Co$^{2+}$, whereas the activity of Nit09 was almost unaffected by the presence of these ions. Interestingly, an increased activity was detected in the presence of ZnCl$_2$. In general, nitrilase activities are independent of metal cofactors (Gong et al. 2012). This was supported by the lack of Nit09 activity inhibition by the presence of the chelating agents EDTA and sodium azide. Thus, a different mechanism like enhanced protein stability in the presence of Zn$^{2+}$ might explain the detected activity increase of Nit09.

The characterization of arylacetonitrilase Nit09 provided additional knowledge on this rare group of nitrilases. The substrate range is common for this enzyme type, but the combination of a low pH optimum, a high temperature

### Table 5 Properties of characterized bacterial arylacetonitrilases

| Organism                               | pH optimum | Temperature optimum (°C) | Molecular mass (kDa) | Accession number | Reference                  |
|----------------------------------------|------------|--------------------------|----------------------|------------------|----------------------------|
| *Alcaligenes faecalis* JM3             | 7.5        | 45                       | 44                   | BAA02684         | Nagasawa et al. (1990)     |
| *Alcaligenes faecalis* ATCC 8750       | 7.5        | 40–45                    | 32                   | CUI34632         | Yamamoto et al. (1992)     |
| *Alcaligenes faecalis* ZJUTB10         | 7.5        | 40                       | 44                   | AEP34036         | Liu et al. (2011)          |
| *Alcaligenes sp.* ECU0401              | 8.0        | 40–45                    | 39                   | ACS13754         | Zhang et al. (2011b)       |
| *Alcaligenes sp.* MTCC 10675           | 6.5        | 50                       | 60                   | AGC11817         | Bhatia et al. (2014)       |
| *Bradyrhizobium diaeofficiens* USDA110 | –          | –                        | 37                   | NP_773042        | Zhu et al. (2007a)         |
| *Burkholderia cenocepa*cia J2315       | 8.0        | 45                       | 37                   | CAR52890         | Wang et al. (2013)         |
| *Burkholderia xenovorans* LB400        | –          | –                        | 39                   | YP_559838        | Seffernick et al. (2009)   |
| *Luminiphilus syltensis* NOR5-1B       | 7.0        | 40                       | 43                   | EED35210         | Sun et al. (2015)          |
| *Pseudomonas fluorescens* EBC191       | 6.5        | 50                       | 38                   | AAW79573         | Kiziak et al. (2005)       |
| *Pseudomonas putida* MTCC 5110         | 7.0        | 40                       | 43                   | ABV21758         | Banerjee et al. (2006)     |
| *Pseudomonas sp.* UW4                  | 6.0        | 50                       | 33                   | AFY19658         | Duca et al. (2014)         |
| *Variovorax boronicumulans* (predicted)| 6.0        | 50                       | 36                   | MN689843         | This study                 |
optimum, and an unusual long-term stability with a pronounced resistance against most metal ions is exceptional. Arylacetonitrilases are industrially interesting biocatalysts as they are currently used for the large-scale conversion of mandelonitrile to mandelic acid (Gong et al. 2012). Thus, identification of new enzymes of this type might open new production routes. Interesting in this respect is the conversion of phenylacetonitrile to phenylacetic acid, which is used among others for the production of penicillin G (Ziemons et al. 2017). The new insights obtained by identification and analysis of Nit09 contribute to the understanding of the sequence function relationship of arylacetonitrilases and thus to the development of better or novel nitrilase biocatalysts.

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Author contributions

RE, RH, and RD conceived the study. IF performed the experiments. RE and IF analyzed the data. All authors interpreted the results, wrote the manuscript, and reviewed the final version of the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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