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Biotransformations of nitriles mediated by *in vivo* nitrile hydratase of *Rhodococcus erythropolis* ATCC 4277 heterologously expressed in *E. coli*

Maraylla I. Moraes [a], César Iglesias [b], Iris S. Teixeira [a], Humberto M.S. Milagre [a], Sonia Rodríguez Giordano [b,*], Cintia D.F. Milagre [a,*]

[a] Institute of Chemistry, UNESP – São Paulo State University, Araraquara, São Paulo, Brazil
[b] Area Microbiología, DEPBIO, Facultad de Química, Universidad de la República, Gral. Flores 2124, Montevideo 11800, Uruguay

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ABSTRACT

Nitrile hydratase activity has been reported as an exciting alternative for the industrial production of a variety of compounds overwhelming its chemical counterpart; despite this, until now, a few enzymes have been thoroughly studied. Efficient expression of nitrile hydratase enzymes has been the bottleneck to explore this activity. Here, we report the cloning and expression of *Rhodococcus erythropolis* ATCC 4277 nitrile hydratase (α- and β-subunits) and the correspondent activator gene. Furthermore, substrate scope with whole cells of recombinant *E. coli* demonstrates that this Fe-type NHase could hydrate a wide range of aliphatic and aromatic nitrile with high conversion rates and moderate enantiomeric excess.

Introduction

Nitrile hydratases (NHase, E C 4.2.1.84) are metalloenzymes that catalyzes the hydration of nitriles to the corresponding amides [1,2]. NHases tridimensional structure consists of two subunits (α- and β-), with a molecular weight of approximately 23 kDa per subunit, and generally exist as αβ dimers or α2β2 tetramers [3,4]. According to the requirement of a metal cofactor, nitrile hydratases can be classified into two types: Fe-type and Co-type. Although they present a highly homologous amino acid sequence along both subunits, Fe- and Co-type NHase differ in photoreactivity, biotransformation activity and substrate specificity [5,6].

Many microorganisms exhibit NHase activity, such as *Rhodococcus equi* TG3282 [7], *Klebsiella oxytoca* KCTC 1686 [8], *Aurantimonas manganoxydans* ATCC BAA-1229 [9], *Pseudomonas chlororaphis* B23 [10] and *Bacillus sp*. BR449 [11] among others. This type of enzyme has attracted considerable attention because of its lucrative application on the industrial production of acrylamide, nicotinamide, 5-cyanovaleramide and pyrazinamide, and for environmental bioremediation [12,13]. These enzymatic routes offer the additional advantage of higher selectivity and greener reaction conditions when compared with traditional ones [14,15].

Rhodococcus erythropolis has been reported to have nitrile hydratase activity [5,15,16]. Indeed, the sequence identity of the enzyme responsible for this activity in *R. erythropolis* JCM6823 has been reported [16]. However, the substrate scope of this enzyme was analyzed with the crude extract of the wild-type strain. An analysis of this strain genome allowed the identification of the genes encoding for the NHase activity; nevertheless, the enzyme was not isolated or expressed heterologously in a different host, and activity of more than one enzyme cannot be overruled. The authors reported that despite the extremely high amino acid sequence similarity among *R. erythropolis* JCM6823 and *Rhodococcus* sp. N-774 NHases, the former presented a broader substrate specificity. Slight changes in protein sequence could explain the differences in substrate scope. Another NHase, from *R. erythropolis* AJ270 has been cloned, successfully expressed in *E. coli* and applied to the synthesis of 3-benzyloyloxy-4-cyanobutyramide and 3-benzyloxy-4-cyanobutyramide [15]. However, the substrate scope of this recombinant strain was not reported. These previous results indicate that the study of microorganisms at the infraspecific level is important in the context of biotechnological discoveries because many sought-after properties are known to be strain-specific as opposed to specie-specific [5].

Regarding the expression of the *Rhodococcus* NHase gene, some reports are available in the literature [12,15,17,18,19]. *E. coli* expression systems remains the preferred ones for large-scale production of recombinant proteins due to the accumulated knowledge, availability of a manifold of commercial systems and large protein productivity [20]. Different expression strategies have been suggested for the NHase gene,

* Corresponding authors.
E-mail addresses: soniar@fq.edu.uy (S.R. Giordano), cintia.milagre@unesp.br (C.D.F. Milagre).

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Table 1
Primers used in this study.

Primer	Sequence	Tm	bp
NHa-F	5′-AGATATACCATGGGCAGCAGC ATGTCAGTAACGATCGACCACAC-3′	53.1	44
NHa-R	5′-TACGATTACTTTCTGTTCGACTTAA GCCCTGGAATGCTGGAAGATCAG-3′	53.7	48
activador-F	5′-TAAGAAGGAGATATACATATGGCAG ATCTCATGGTCGACACACGACTTCC-3′	52.0	50
activador-R	5′-CTCAGCGGTGGCAGCACAATA TCAAACGGTCTGGTCG-3′	52.2	37
DuetUp2	5′-TTGTACACGGCCGCATAATC-3′	57.3	20
T7 terminator	5′-GCTAGTTATTGCTCAGCGG-3′	54.5	19
pACYCDuetUp1	5′-GGATCTCGACGTTCTCCCT-3′	61.0	19
DuetDown1	5′-GATTATGCGGCCGTGTACAA-3′	57.3	20

encompassing the use of multiple expression vectors or a single vector with independent promoters. Song *et al*. reported an expression system, where the α and β subunits shared the same promoter while the activator gene was controlled by a different promoter. Verseck *et al*. investigated the expression of the *R. erythropolis* 870-AN019 NHase gene in a two-vector expression system, in which the β-subunit was cloned into pET26b while the α subunit and the activator gene (p47K) were cloned together in pET22b [21].

Therefore, the search for novel NHases, as well as adequate strategies for the construction of recombinant biocatalysts expressing these enzymes continues to be a goal worth pursuing. In this study, the genes encoding the α- and β-subunits of *R. erythropolis* ATCC 4277 NHase and the correspondent activator gene were cloned and functionally expressed in *E. coli* BL21 (DE3). The whole cells of recombinant *E. coli* were used as a biocatalyst for the *in vivo* transformation of aliphatic and aromatic nitriles to the respective amides, with the objective of evaluating their substrate scope, regio- and stereoselectivity.

Materials and methods

Chemicals

The acrylonitrile (**1a**) was donated by the chemical company (Aratrop Industrial Company, Brazil). Butyronitrile (**2a**), isobutyronitrile (**3a**), benzonitrile (**4a**), o-tolunitrile (**5a**), 1,4-phenylenediacetonitrile (**6a**), 2-phenylbutyronitrile (**7a**), benzoylacetonitrile (**8a**) and 4-cyanopyridine (**9a**) were purchased from Sigma-Aldrich. Potassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), bovine serum albumin (BSA) and isopropyl β-D-1-thiogalactopyranoside (IPTG) were also purchased from Sigma-Aldrich. Both hexane and 2-propanol HPLC grade were acquired from Honeywell Research Chemicals. All solvents were used without further purification.

Plasmids, strains and growth conditions

Plasmid pACYCDuet-1 was purchased from Novagen (Darmstadt, Germany) and was used as the expression vector. The NHase genes were cloned from *Rhodococcus erythropolis* ATCC 4277 (CCT 1878, obtained from Tropical Culture Collection, André Tosello Foundation, Brazil). *Escherichia coli* JM109 was used for recombinant plasmid construction and *E. coli* BL21 (DE3) was used as the host for recombinant expression in this study. *E. coli* JM109 and BL21 (DE3) cells were obtained from New England Biolabs (Ipswich, MA, USA). These strains were cultivated at 37 °C in Luria–Bertani (LB) broth or on LB agar plates, supplemented with chloramphenicol when necessary (34 μg/mL).

Amplification of genes

From the nucleotide sequence encoding NHase from *Rhodococcus erythropolis* (UniProtKB – Q6XMT1, Q6XMT2 and B1GY04), specific primers were designed and synthesized (Macrogen, Korea). The primers used in this study were listed in Table 1.

The activator gene was cloned using primers activator-F and activator-R and named as NH-AT1. The primers NHa-F and NHa-R were used to amplify the DNA fragment encoding the α- and β-subunits of NHase and named as NH-AB1. PCRs were performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs). The amplification conditions comprised initial denaturation at 98 °C for 30 s, followed by 30 cycles of denaturation (98 °C, 10 s), annealing (64 °C, 30 s), and extension (72 °C, 120 s) with a final extension step at 72 °C for 10 min. The PCR products were analyzed by 0.8 % agarose gel electrophoresis and purified using QIAquick Gel Extraction Kit (Qiagen). Purified products (megaprimers) were used in subsequent RF amplification.

RF cloning amplification reaction

The second RF PCR was performed using 0.2 mL PCR tubes in a final volume of 100 μL including the following components: 30 ng of target plasmid (pACYCDuet-1), 120 ng of PCR product (NH-AT1), 200 μM of each dNTPs, 5X Phusion HF buffer and Phusion High-Fidelity DNA Polymerase. The PCR product was treated with *Dpn*I for 24 h at 37 °C to digest the methylated parental plasmid, followed by precipitation with yeast tRNA to enhance transformation efficiency [22]. The product was then transformed into *E. coli* JM109 competent cells and positive clones were confirmed by PCR using the primer activator-F and activator-R. The recombinant plasmid was purified using PureLink® HiPure kit (Invitrogen) and named pACYCDuet-NH-AT.

To clone the α and β subunits, 120 ng of PCR product (NH-AB1) was used in a RFcloning amplification reaction with 30 ng of pACYCDuet-NH-AT, 200 μM of each dNTPs, 5X Phusion HF buffer and Phusion High-Fidelity DNA Polymerase in a total volume of 100 μL. The PCR program was as follows: 1 cycle at 98 °C for 30 s; 30 cycles of 98 °C for 10 s, 62 °C for 30 s, and 72 °C for 120 s; and a final extension step at 72 °C for 10 min. The PCR product was treated with *Dpn*I, precipitated with yeast tRNA and transformed into *E. coli* JM109 competent cells. Positive clones were confirmed by DNA sequencing (Macrogen Inc. Korea). The recombinant plasmid was designated pACYCDuet-NH-RE. Competent cells of *E. coli* BL21(DE3) were transformed to obtain the recombinant strain *E. coli* BL21(DE3) (pACYCDuet-NH-RE) for NHase expression.

Sequence analysis

The obtained DNA sequences were analyzed and translated using Vector NTI software package (Invitrogen). Alignments of nucleotide and amino acid sequences were performed using Basic Local Alignment Search Tool (BLAST) or ClustalX 2.1 version [23,24].

Expression of recombinant NHase in E. coli

Fresh plates of *E. coli* BL21(DE3) (pACYCDuet-NH-RE) were streaked from frozen stocks, and a single colony was used to inoculate 5 mL of LB-chloramphenicol. The culture was incubated in a rotary shaker overnight (200 rpm, 37 °C), and 1 mL of this culture was used to inoculate 100 mL of fresh LB- chloramphenicol in a 500 mL Erlenmeyer flask. The fresh culture was grown under the same conditions until it reached an OD_{600} of 0.5 and then IPTG was added to a final concentration of 1 mM. After 24 h induction at 20 °C, recombinant *E. coli* cells were harvested by centrifugation and used for resting cells biotransformation reactions.

Enzymatic activity determination

The nitrile hydratase activity was measured using a modified Mauger *et al* method [25]. NHase activity in the culture medium was assayed in a 1 mL reaction mixture containing KH_2PO_4/K_2HPO_4 buffer (0.1 M, pH = 7.5), benzonitrile (9.7 μmol) and *E. coli* whole cells (100 mg of wet weight). The reaction was incubated at 28 °C for 5 min and was stopped

with addition of 0.1 mL of 1 M HCl. Cells were removed by centrifugation at 5,000 × g for 10 min. The amount of benzamide was determined by Gas Chromatography with Flame Ionization Detector (GC-FID). The specific activity of the benzamide production was defined as the amount of cells in milligrams that catalyzes the formation of 1 μmol of benzamide per minute.

General procedure for biotransformation

The biotransformation reactions with recombinant NHase were carried out using several aliphatic and aromatic nitriles (Table 1). Cell pellets (~1.5 g wet weight) were resuspended in 50 mL of KH_2PO_4/K_2HPO_4 buffer (0.1 M, pH = 7.5) containing substrate (0.2 mM) and dimethyl sulfoxide (0.05 % v/v) as cosolvent to help in the substrate solubilization. Reactions were maintained at 28 °C and 150 rpm under orbital shaking for 24 h. Cells were removed by centrifugation at 3200 rpm for 15 min and the supernatant was extracted with ethyl acetate (3x), dried with anhydrous magnesium sulfate, and analyzed by GC-FID. In parallel, blank experiments were performed with recombinant cells without substrate.

SDS-page and protein determination

The expression of the recombinant NHase was analyzed using SDS-PAGE (15 %) with a 4 % stacking gel. Recombinant cells (1 mL) were collected by centrifugation at 12,000× g for 10 min and the pellets resuspended in 300 μL lysis buffer (pH 8.5). Ten microliters of lysozyme (5 mg/ml) were added, and the sample was incubated for 15 min to break down the cell walls, followed by the addition of 2 μL of Triton® X-100 (10 %). The supernatant (soluble proteins) and cell pellet (insoluble proteins) were obtained by centrifugation at 12,000× g for 10 min at 4 °C and analyzed SDS-PAGE. Protein concentration was determined according to Bradford using bovine serum albumin as a standard [26].

Nucleotide sequence accession numbers

The gene sequences of α-subunit, β-subunit and the activator of NHase from *R. erythropolis* ATCC 4277 have been deposited in GenBank (GenBank accession number: MH732727, MH732728, and MH732729).

Analytical methods

Chemical reactions were monitored by gas chromatography – flame ionization detector (GC-2010 Plus, Shimadzu) with an Rtx-5® (95 % dimethylpolysiloxane and 5 % diphenyl) capillary column (30 m × 0.25 mm × 0.25 μm) under the following conditions: injector temperature, 260 °C; detector temperature, 280 °C; hydrogen flow rate, 1.22 mL/min and the column oven temperature program was 80 °C hold for 3 min, raised 30 °C/min to 280 °C, 280 °C hold for 5 min.

The enantiomeric excess of 2-phenylbutyramide (**7b**) was determined by high performance liquid chromatography (HPLC, Jasco LC-NetII/ADC, equipped with photodiode array (MD-2018 Plus) using Chiralcel OD-H column (Daicel) with a mobile phase consisting of 2-propanol: hexane, 9:1 (v/v) at a flow rate of 0.8 mL/min. The products **3b** and **6b-8b** were characterized by their ^1H and ^{13}C NMR spectra recorded on spectrometer Bruker Avance III HD 600. ^1H and ^{13}C NMR spectra were carried out in $CDCl_3$ (with TMS as an internal standard) or in CD_3OD on a Bruker 300 Fourier (7,1 T) spectrometer. Chemical shifts (δ) are recorded in ppm and spin–spin coupling constant (*J*) in Hz. Multiplicities are reported by the following abbreviations: s = singlet, d = doublet, t = triplet, m = multiplet, q = quartet, and br s = broad singlet.

The products **2b**, **4b**, **5b,** and **9b** were characterized by gas chromatography with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm, (5 %-Phenyl)-methylpolysiloxane) coupled to a mass spectrometer. GC–MS analyses were recorded on an Agilent 7890B gas chromatograph (GC) coupled to a 5977A mass spectrometer (MS). Helium carrier gas was set at 1 mL/min with constant flow mode, injector at 260 °C set in split mode (100:1), injection in the volume of 1 μL and sample concentration in the range of 0.5 – 1.0 mg/mL. The GC oven temperature programing is as follow: 80 °C hold for 3 min, raised 30 °C/min to 280 °C, 280 °C hold for 3 min. Mass spectrometer transfer line into the quadrupole was set at 280 °C, ionization voltage of 70 eV and mass spectra were obtained in full scan mode (40 – 400 *m/z*).

Polarimetry was carried out using a Perkin Elmer Model 341 Polarimeter (measurements being made at the sodium D-line) with a 1.0 dm pathlength cell and concentrations (*c*) in g/100 mL. The absolute configuration of **7b** was determined by comparison of $[\alpha]_D$ value from the literature [27,28].

Procedure for chemical synthesis of amides 2b-8b

In a round-bottom flask, 1 mmol of the nitrile (**2a-8a**) was solubilized in 4 mL of anhydrous CH_2Cl_2. After complete solubilization, H_2SO_4 was added dropwise to the reaction medium, under slow magnetic stirring. The reaction was stirred at room temperature and monitored by GC-FID. After completion, the reaction was quenched by addition of NaOH 10 M until pH 8–10 followed by repeated (3x) extraction with equal volumes of ethyl acetate (EtOAc). The organic phases were combined, dried under anhydrous magnesium sulfate, and the solvent was evaporated.

Procedure for chemical synthesis of 4-pyridinecarboxamide (9b) [29]

In a round-bottom flask 4-cyanopyridine **9a** (1 mmol; 104 mg) was solubilized in 5 mL of Etanol-H_2O (1:1) and sodium borohydride (0.75 mmol; 28.4 mg) was added. The reaction solution was stirred at 80 °C for 2 h and monitored by GC-FID. After completion, the reaction mixture was concentrated in the rotaevaporator, the residue was solubilized in aqueous phase and the aqueous phase extracted with EtOAc (3×5 mL). The organic phases were combined, dried under anhydrous magnesium sulfate, and the solvent was evaporated.

Bioinformatic analysis

The AlphaFold models were calculated with the Google Colab platform and AlphaFold2_advanced option and refined using the Amber-relax option to enhance the accuracy of the side chains geometry, only the best model among the five best given by default was examined in detail [30]. Structural alignments were generated using THESUS algoritm in YASARA software [31].

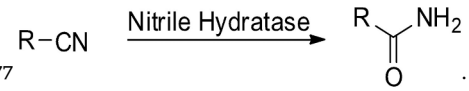
Results

Cloning and expression of the nitrile hydratase genes of Rhodococcus erythropolis ATCC 4277

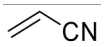
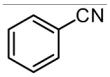
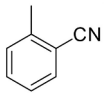
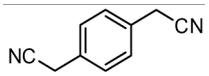
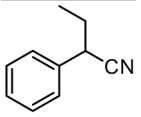
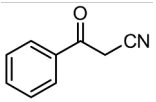
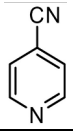
In this work, the genes encoding NHase from *R. erythropolis* ATCC 4277 were inserted into the expression vector pACYCDuet-1 (4008 bp) (Fig S1 - Supporting Information). This vector allows the co-expression of two target genes under control of separate T7*lac* promoters [32]. We devised a strategy in which the activator is expressed from one of the T7*lac* promoter, while the other acts as a *cis* element for the expression of both the α- and β NHase subunits. For construction of the recombinant plasmid pACYCDuet-NH-RE, the genes were amplified by PCR using specific primers for the *in vitro* recombination of the genes and the vector according to the RFcloning procedure [33]. The primers activator-F and activator-R (Table 1) were used to amplify a 1200 bp PCR product, encoding the activator gene. The purified PCR product was used as megaprimer to amplify the pACYCDuet vector, yielding the vector pACYCDuet-NH-AT. The oligonucleotides NHa-F and NHa-R were used to amplify a 1263 bp PCR product encoding both the α- and β-subunits. The fragment was used as megaprimer to amplify pACYCDuet-NH-AT

Table 2

Substrate specificity of NHase from *R. erythropolis* ATCC 4277



(**a**) (**b**)

Entry	Nitrile	Structure	Conversion[a] (1 % DMSO) %	*ee*
1	Acrylonitrile (**1a**)		0	
2	Butyronitrile (**2a**)		>99	
3	Isobutyronitrile (**3a**)		>99	
4	Benzonitrile (**4a**)		>99	
5	o-Tolunitrile (**5a**)		>99	
6	1,4-Phenylenediacetonitrile (**6a**)		99	
7	2-phenylbutyronitrile (**7a**)		99	60[b] (*S*)-selectivity
8	Benzoylacetonitrile (**8a**)		33	
9	4-cyanopyridine (**9a**)		9	

a- conversion determined by GC-FID.
b- determined by HPLC.

yielding pACYCDuet-NH-RE (Fig S2 - Supporting Information). The resultant construction was verified by sequencing and transformed into *E. coli* BL21(DE3). The recombinant strain was used for protein expression based on a traditional protocol, with the induction temperature reduced to 20 °C to avoid aggregation of the recombinant NHase. Expression of the α- and β-subunit (~23 kDa) as well as the activator (~45 kDa) was verified on SDS-PAGE gel (Fig S3 - Supporting Information). Since α- and β-subunits have similar size, it was not possible to assess the individual level of expression from the SDS-PAGE, as it was already reported by Song *et al* [15]. The specific activity from the whole cells of *E. coli* (pACYCDuet-NH-RE) was 0,0011 U/mg cells. The effective expression of the *R. erythropolis* ATCC 4277 NHase was evident from the SDS electrophoresis as well as from the activity assay, thus indicating that α- and β- subunits were co-expressed as an operon, under control of just one T7 promoter.

Substrate specificity and stereoselectivity

The whole cells of recombinant *E. coli* BL21(DE3) (pACYCDuet-NH-RE) were used in resting cell biotransformation reactions to evaluate the substrate specificity of the biocatalyst, and the results are given in Table 2. The recombinant NHase from *R. erythropolis* ATCC 4277 was efficient in hydration of aliphatic nitriles, such as butyronitrile (**2a**) and isobutyronitrile (**3a**), with conversions higher than 99 % in the formation of butyramide (**2b**) and isobutyramide (**3b**), respectively. On the other hand, no activity was found for acrylonitrile (**1a**). Additionally, most of the tested aromatic compounds were hydrated with high conversion rates, varying from excellent for **4a-7a** to low for **8a-9a**. The conversion of benzonitrile (**4a**) into benzamide (**4b**) was higher than 99 %; however, only 9 % conversion was achieved for 4-cyanopyridine (**9a**) (Table 2, entries 4 and 9). The relevance of some steric hindrance was evaluated by the inclusion of *o*-tolunitrile (**5a**) among the tested substrates resulting in over 99 % conversion to the respective o-toluamide (**5b**). Bulkier substrates were also assayed (**6a – 8a**), yielding good to excellent conversion. Inclusion of 2-phenylbutyronitrile (**7a**), a prochiral substrate, allowed a preliminary evaluation of the enzyme stereoselectivity, yielding (*S*)-(+)-2-phenylbutyramide (**7b**) in 99 % yield and 60 % enantiomeric excess of in 24 h.

Analysis of the R. erythropolis ATCC 4277 NHase sequence and three-dimensional structure

A BLAST search based on NCBI database was performed for both, the α- and β- subunits. The α-subunit shared homology with Fe-type NHase from *Bacillus* sp. NN1 (100 %), *Paenibacillus* sp. NN32 (100 %), *Rhodococcus* sp. NN5a (99 %), *Microbacterium* sp. AJ115 (98 %) and *Gordonia polyisoprenivorans* (83 %). Similarly, *R. erythropolis* ATCC 4277 β-subunit exhibited 97 % homology with the amino acid sequence from *Bacillus* sp. SW2-21, 93 % identity with *Microbacterium* sp. SW2-8 and 88 % identity with *Rhodococcus opacus*.

Although the α-subunit is highly conserved among different *Rhodococcus* NHases (Fig S4 - Supporting Information), several differences were registered between BAA 03348.1 (*R. erythropolis* JCM 6823) and the one reported herein. Differences in several positions were detected, encompassing G22R, D58A, E69D, R86G, I87Y, S88L, R92G, D100R, P181G and Q184R. Particularly interesting is the change in the

aminoacidic triad 86, 87 and 88. A comparison of the β-subunit presented similar results, with these two enzymes differing in six positions: P71S, T82A, P117R, E119D, S154A, K183T. The relevance of the β-subunit for the catalytic activity is revealed by the fact that all known NHases have a β-subunit or homologous subunit involved in the active site structure [34]. A highly conserved hydrogen bonding network links the two subunits, including linkages between post-translationally modified cysteine residues of the α-subunit with the two arginine residues of the β-subunit that, when mutated, dramatically reduce the activity, or suppress it completely [34,35].

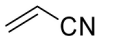
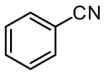
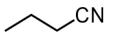
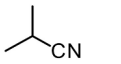
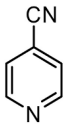
In order to correlate the assessed differences on substrate specificity with these small variations in aminoacidic sequence, computational models were created for *R. erythropolis* ATCC 4277 and for *R. erythropolis* JCM 6823 NHases using alpha fold [29,36]. Additionally, the crystallized *R. erythropolis* NHase PDB 2D0Q was analyzed to help localize the active site, since this NHase has been crystallized with cyclohexylisocyanide as a ligand [37]. The obtained enzyme models were analyzed by superimposing the two characterized variants.

Discussion

Previous work with *Rhodococcus* NHases has shown that the genes encoding this enzymatic activity are present in an operon, thus its expression under control of a unique promoter is likely to occur. However, previous attempts to express *K. oxytoca* α- and β-subunits, as well as the activator, on the same vector under control of one promoter have failed [8], despite the close relationship among *K. oxytoca* and *E. coli*. A previous work by Song and coworkers has shown that *R. erythropolis* NHase could be expressed from a unique vector construction, with the activator protein expressed from a different promoter [15]. From this work, recognition of *Rhodococcus* ribosome binding site (RBS) by the *E. coli* host seemed possible, although some other authors have indicated that genes as well as RBS optimization could be necessary to achieve expression [38]. The effective expression of the *R. erythropolis* ATCC 4277 NHase was evident from the SDS electrophoresis as well as from the activity assay, thus indicating that α- and β- subunits were co-expressed as an operon, under control of just one T7 promoter, indicating that *E. coli* can recognize the *R. erythropolis* RBS for translation of the β subunit despite being distantly related. Co-expression of the activator gene was possible under control of a separate T7 promoter, in agreement with previous results [15]. Additionally, the restriction-free (RF) cloning method was an effective choice for cloning the *R. erythropolis* ATCC 4277 NHase genes since independence of restriction sites enormously simplify the cloning procedure [33].

An analysis of the *R. erythropolis* ATCC 4277 α-subunit aminoacidic sequence revealed the presence of the characteristic iron-binding motif, CSLCSC, which differs from the cobalt-binding standard, CTLCSC. According to the literature, Fe-type NHases preferentially catalyze the hydration of aliphatic substrates, while Co-type NHases usually convert aromatic and aliphatic compounds [7,39]. However, it has been reported that several Fe-type nitrile hydratases exhibit wide substrate specificity, such as NHases from *Rhodococcus equi* TG328-2, *Pseudomonas putida* F1 and *R. erythropolis* A4 [3,6,40]. The Fe-type NHase of *Rhodococcus erythropolis* ATCC 4277 does follow this trend, as high conversions were obtained for a broad range of aliphatic and aromatic nitriles with no preference for the aliphatic or aromatic ones (Table 2). The recombinant NHase from *R. erythropolis* ATCC 4277 was active for the hydration of some aliphatic nitriles such as (**2a**) and (**3a**), as well as aromatic ones, varying from excellent activity for **4a-7a** to poor activity for **8a-9a**. On the other hand, no activity was found for acrylonitrile (**1a**) which agrees with Endo *et al.* that reported Co-type NHases as more stable and efficient to produce acrylamide than Fe-type ones [41]. As it become clear from Table 2, the NHase activity is highly dependent on substrate structure. While the neutral benzonitrile (**4a**) is converted into benzamide (**4b**) in a very high yield, the conversion of the basic related 4-cyanopyridine (**9a**) was very poor (Table 2, entries 4 and 9), showing

Table 3
Comparative analysis of the substrate specificity among *R. erythropolis* JCM6823 NHase and the one reported herein (*R. erythropolis* ATCC 4277) (a qualitative comparison was performed since activity has been reported in different ways, -, +, ++ and +++ for none, scarce, good and very good activity).

Aliphatic substrates			Aromatic substrates		
substrate	This work	JCM6823		This work	JCM6823
	–	++		+++	+++
	+++	++			
	+++	–		+	+++

that this difference in neutral-basic feature may have an important effect on reactivity. In the case of *o*-tolunitrile (**5a**) conversion to the respective o-toluamide (**5b**) was also higher than 99 %, indicating that the increase in steric hindrance next to the nitrile group did not affect the reaction. The presence of a methylene spacer did not affect activity neither, as become evident from the high conversion of 1,4-phenylenetrietonitrile (**6a**) to 4-(cyanomethyl) benzeneacetamide (**6b**). An interesting feature of this biotransformation was the strict regioselectivity since only the corresponding monoamide was obtained, in agreement with previous reports for *Rhodococcus* ATCC BAA-870 NHase, although conversion rates were not disclosed for this enzyme [42].

Surprisingly, the (*S*)-(+)-2-phenylbutyramide (**7b**) was obtained as the product of the transformation of 2-phenylbutyronitrile (**7a**), with a conversion of 99 %, and a moderate and unexpected enantiomeric excess of 60 % in 24 h. Previously, NHases were assumed to be relatively non-stereospecific [8,34]; however, recently, some enantioselective NHases from *Rhodococcus equi* A4 [39], *Agrobacterium tumefaciens* d3 [43], *Pseudomonas putida* NRRL-18668 [44], *Rhodococcus* sp. AJ270 [45] and *Klebsiella oxytoca* [46] have been reported. While Prepechalová *et al.* observed that the nitrile hydratase from *R. equi* A4 was not stereoselective at all in the hydration of (**7a**) to (**7b**) [39], Wang *et al.* determined that *Rhodococcus* sp. AJ270 NHase was stereospecific on the hydration of substrate (**7a**) yielding the (*R*)-(-)-**7b** enantiomer in 96 % *ee* and 34 % conversion after 96 h [27]. The hydration of (**7a**) by *Rhodococcus* sp. (SP 361) was observed by Beard *et al.* with 31 % yield and 90 % enantiomeric excess of (*R*)-(-)-**7b** in 71 h [28]. Rzeznicka *et al.* reported that the nitrile hydratase from *Rhodococcus equi* TG328-2 hydrated compound (**7a**) with a conversion of 51 % and enantiomeric ratio (E) = 12 [7]. Unfortunately, the authors did not report the stereochemistry of the product. Nevertheless, as a rule of thumb, E below 15 are unacceptable for practical purposes [39]. The observed *ee* of NHase from *R. erythropolis* ATCC 4277 studied herein is certainly not satisfying yet but is a good starting point for process or protein engineering, overall, when it presents the opposite stereochemistry than that described for other NHases.

To help understand the structural motif associated with substrate specificity, a comparative analysis was performed among *R. erythropolis* JCM6823 NHase and the one reported herein, *R. erythropolis* ATCC 4277). Although these enzymes are highly conserved, small changes in amino acids may be responsible for the difference in substrate specificity for these two nitrilases (Table 3).

As is become evident from the comparative analysis, our results with the aliphatic substrates largely differ from those obtained with the nitrile hydratase from *R. erythropolis* JCM 6823, that present very good activity on acrylonitrile (**1a**) and almost no activity on isobutyronitrile (**3a**) [16]. Additionally, an important difference has also been registered when using 4-cyanopyridine (**9a**) as a substrate. Unfortunately, there is

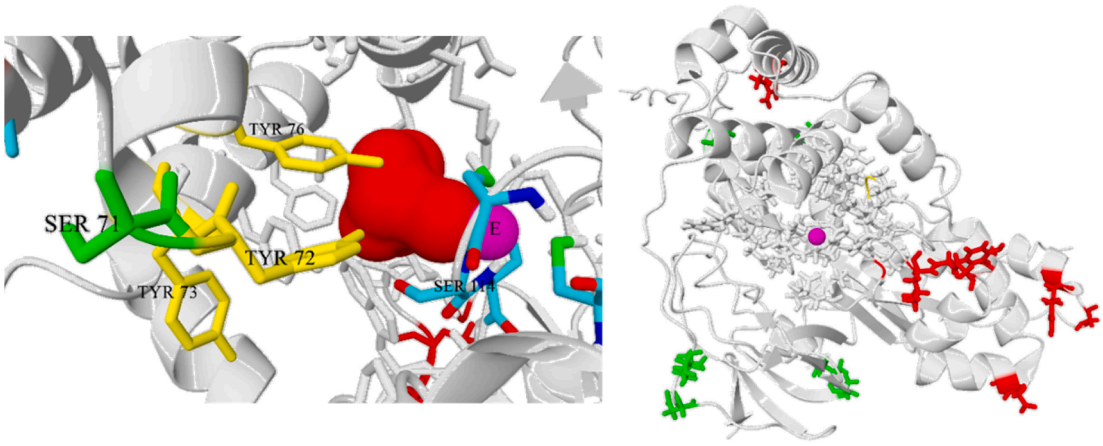


Fig. 1. Left: The amino acid serine 71 is shown in green, adjacent tyrosine 72 and 76 enter into the structure and participate directly in the active site of the protein. Right: As can be seen in the figure (green and red), changes in this variant have no direct impact on the active site (Fe in purple) .

no data available to compare a larger set of substrates.

In order to correlate the assessed differences on substrate specificity with the small variations in aminoacidic sequence, the obtained enzyme models were analyzed by superimposing the two characterized variants. The overall comparison of both structures shows that there is no major structural difference among these two nitrile hydratases, thus a deeper analysis of variations in protein structure in the tunnel of access and the active site were explored. As can be seen in Fig. 1, our variant has a serine at position 71 of the beta subunit instead of proline. This position is located in the loop that starts the alpha helix involving the amino acids Tyr72 and Tyr76 which are directly involved in the active site and are located at distances of less than 4 A° from the catalytic iron and the amino acid serine 114 (involved in the mechanism). The change from proline to serine in this variant could influence the disposition of the above mentioned tyrosines that penetrate the active site, and this might explain the differences in substrate specificity associated to slight changes in the substrate. Furthermore, the metal atom is located in the central cavity formed by the surfaces of the two different subunits. The entrance tunnel is also defined at this interface, and substrate access to the active site is ensured by dynamic oscillations of the protein structure during catalysis, thus, differences in residues far away from the active site could be affecting the tunnel access space, being another possible explanation for the differences in activity observed in among these two variants [47].

Conclusions

In summary, a new biocatalyst with NHase activity was constructed that accepts both aliphatic and aromatic nitriles. Despite the high similarity in amino acid sequence with previously reported NHases, the *R. erythropolis* ATCC 4277 NHase showed different substrate specificity when compared to previously reported ones indicating that a limited number of substitutions at particular amino acid residues in the α- and β-subunits may account for the differences in substrate specificity. Additionally, the novel biocatalyst presented moderate stereoselectivity with a tested substrate, differing in stereochemistry from previously reported ones. This finding could set the bases for further analysis and protein engineering experiments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rechem.2022.100760.

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