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Enantioselective conversion of α-arylnitriles by *Klebsiella oxytoca*

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ABSTRACT

A new bacterial isolate *Klebsiella oxytoca* 38.1.2 with stereoselective nitrile hydratase activity against *rac*-2-phenylglycine nitrile, *rac*-2-phenylpropionitrile and *rac*-mandelonitrile was investigated. The cultivation conditions for growth and nitrile hydratase formation were studied. An intracellular (*S*)-enantioselective nitrile hydratase and a putative (*S*)-selective amidase were found to be induced in the presence of *rac*-2-phenylpropionitrile. The temperature dependence on the enantioselectivity of the nitrile hydratase active cells was studied in more detail for the biotransformation of *rac*-2-phenylpropionitrile and *rac*-mandelonitrile. By increasing the temperature from 15 °C to 37 °C, the apparent enantiomeric ratio of the conversion of *rac*-2-phenylpropionitrile to (*S*)-2-phenylpropionamide increased from 16 to 35 at nearly 50% conversion rate. *rac*-Mandelonitrile was converted to (*S*)-mandelamide with an enantiomeric excess of up to 95% in a 80% yield without further conversion to mandelic acid.

1. Introduction

Nitriles are widely used in the organic synthesis of compounds such as amides and carboxylic acids. In the past two decades, the enzymatic hydrolysis of nitriles has been recognized as a method to afford a broad spectrum of useful amides and carboxylic acids. The enzymatic hydrolysis of nitriles follows two possible pathways.[1] Nitrilases (EC 3.5.5.1) catalyze the conversion of nitriles to the corresponding acids directly, whereas nitrile hydratases (NHase, EC 4.2.1.84) catalyze the hydration of nitriles to their amides, which subsequently might be converted to the corresponding carboxylic acids by amidases (EC 3.5.1.4).

Despite the fact that early experiments suggested low enantioselectivity for nitrile converting enzymes,[2] more and more reports succeeded in the enantioselective bioconversion of nitriles. One of the first described enantioselective NHases was isolated from *Pseudomonas putida* NRRL-18668. This cobalt containing NHase catalyzed the hydration of (*S*)-2-(4′-chlorophenyl)-3-methylbutyronitrile at least 50 times faster than that of (*R*)-2-(4′-chlorophenyl)-3-methylbutyronitrile.[3] The NHase of *Agrobacterium tumefaciens* d3 hydrated various 2-arylpropionitriles (2-phenylpropionitrile, 2-phenylbutyronitrile and ketoprofen nitrile) and other aromatic and heterocyclic nitriles enantioselectively to the respective (*S*)-amide. For the (*S*)-phenylpropionamide an ee value above 90% was observed until about 30% of the respective substrate was converted.[4] The purified NHase of *Rhodococcus equi* A4 preferentially hydrated the (*S*)-isomers of racemic 2-(4-methoxyphenyl)propionitrile, 2-(4-chlorophenyl)propionitrile and 2-(6-methoxynaphthyl)propionitrile (naproxen nitrile) with enantiomeric ratios (*E*-values) of 5–15.[5] Recently, we investigated new whole cell biocatalysts with enantioselective activities of NHases as well as amidases. The whole cell isolates selectively converted the (*S*)-enantiomer of *rac*-phenylglycine nitrile with *E*-values of 1.2–5.4.[6]

Regarding NHases from *Klebsiella*, only a few reports are present. Nawaz et al.[7,8] reported on a strain of *Klebsiella pneumoniae* capable of using aliphatic and aromatic nitriles as the sole source of nitrogen as a promising strain for the bioremediation of sites contaminated with nitriles. Hooru et al.[9] cloned and expressed the gene for NHase of *Klebsiella* MCI 2609 in *Escherichia coli*. They demonstrated the conversion of acrylonitrile to acrylamide by this transgenic *E. coli*. However, to our knowledge, no study has been devoted to an enantioselective NHase from *Klebsiella*.

Herein, we report data of a bacterial isolate from *Klebsiella oxytoca*, which demonstrate the enantioselective conversion of racemic α-arylnitriles to their amide products. We further present the cultivation procedure for the production of the nitrile hydratase from *K. oxytoca* strain 38.1.2. The temperature dependence of the enantioselectivity was investigated for the conversion of three α-arylnitriles: 2-phenylglycine nitrile (PGN), 2-phenylpropionitrile (PPN) and mandelonitrile (MN).

2. Results and discussion

2.1. Production of NHase from *K. oxytoca* 38.1.2

In a screening, different bacterial strains with NHase activity were selected. Strain 38.1.2 was shown to convert racemic mandelonitrile with high enantioselectivity (ee = 89%) to (*S*)-mandel- amide (MAm). After identification by 16S-rDNA sequence analysis, the strain was approved as *K. oxytoca*.

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For the optimization of bacterial growth, several carbon and nitrogen sources were tested. Using a defined mineral medium containing fructose as a carbon source and 2-phenylpropionitrile as a sole source of nitrogen, the growth rate was only 0.03 h^{-1}. The optimal cell growth was reached with glucose and ammonium sulfate. The production of NHase was induced by addition of 1 mM 2-phenylpropionitrile. The best results were obtained when induction was done at 100% depletion of the nitrogen source. The induction of NHase in the presence of ammonia was not possible. Bauer et al.[10] also reported the repression of the synthesis of NHase activities in *A. tumefaciens* d3 by ammonia.

A shaking flask cultivation was carried out in a 550 mL scale. The course of the cultivation is shown in Figure 1. The maximum growth rate was 0.3 h^{-1} until the time of induction. After induction, the growth rate decreased to 0.04 h^{-1}. The production of NHase started 4 h after addition of 2-phenylpropionitrile, which could be also detected by the conversion of 2-phenylpropionitrile to 2-phenylpropionamide (PPAm). The maximum activity was reached 7 h after addition of 2-phenylpropionitrile resulting in NHase activity of 9 μkatL^{-1}. At the same time, 2-phenylpropionitrile was totally converted. With further conversion of 2-phenylpropionamide to 2-phenylpropionic acid (PPAc), the NHase activity began to decrease. At the time of the maximum activity, the cultivation process yielded a biomass of 2.6 g dry mass L^{-1} and a NHase activity of about 3.5 μkat g^{-1} dry mass.

The NHase of *K. oxytoca* 38.1.2 seemed to be co-induced by the amide, as the activity arose with increasing amide concentration in the culture medium. This was assured by the fact that the strain showed NHase activity when it was cultured with 2-phenylpropionamide as a sole source of nitrogen (data not shown). Komeda et al.[11] showed that the low molecular mass NHase from *Rhodococcus rhodochrous* J1 is induced by its reaction product, the amide. In their study, genes required for the amide-dependent induction were identified. Mayaux et al.[12,13] showed the structural evidence for genetic coupling of amidases with NHases.

2.2. Conversion of (*RS*)-phenylglycine nitrile

Whole cells of *K. oxytoca* were incubated with (*RS*)-phenylglycine nitrile in 1 mL scale at pH 7.4, and a sample was analyzed after 20 min by chiral HPLC. The NHase preferentially converted the (*S*)-enantiomer to (*S*)-phenylglycine amide with an *E*-value of 7.8.

For the demonstration of the time-dependent effects in the stereoselective biocatalyst reaction, the time course of hydrolysis of (*RS*)-phenylglycine nitrile using permeabilized cells of *K. oxytoca* is shown in Figure 2. In the initial phase of the reaction, (*S*)-phenylglycine nitrile was converted six times faster than (*R*)-phenylgly-

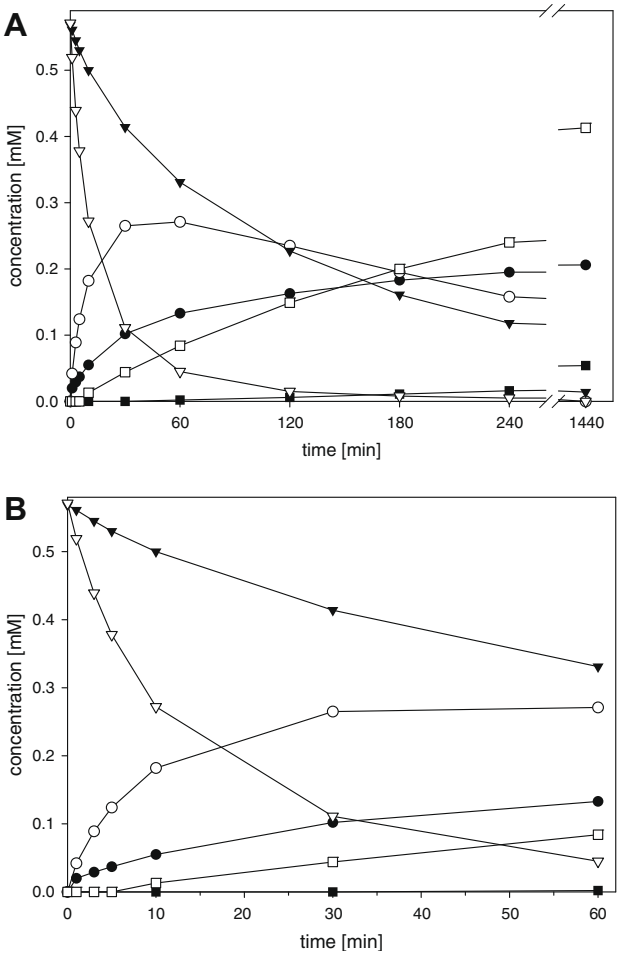

Figure 2. Conversion of (*RS*)-phenylglycine nitrile by permeabilized cells of *Klebsiella oxytoca* 38.1.2 (30 °C, 40 mL-scale). The concentrations of phenylglycine nitrile ((*R*)-▼ and (*S*)-▽), phenylglycine amide ((*R*)-● and (*S*)-○) and phenylglycine ((*R*)-■ and (*S*)-□) were monitored. In **A,** the total course of the conversion is shown, whereas in **B** only the course of the first 60 min is displayed.

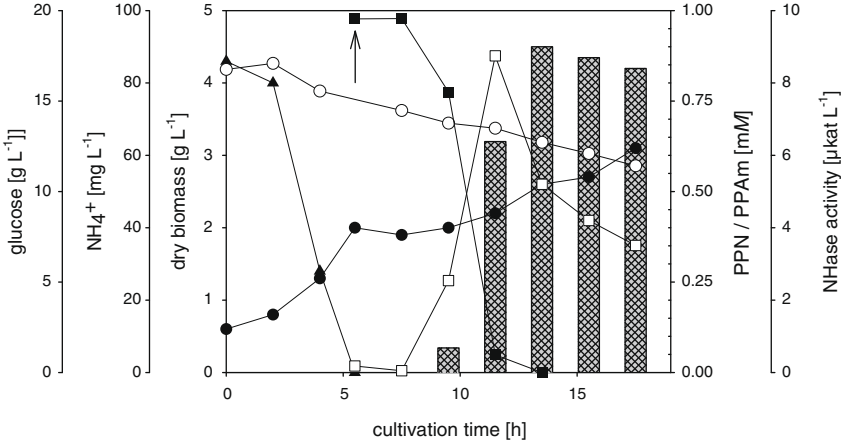

Figure 1. Production of the nitrile hydratase (NHase) from *Klebsiella oxytoca* 38.1.2. The time course of the shaking flask cultivation (550 mL-scale) in a synthetic medium with glucose as carbon source and ammonium sulfate as a nitrogen source is shown. The cell growth (cell dry mass, ●), the NHase activity (▒), and the glucose (○), PPN (■), PPAm (□) and ammonia (▲) concentration were monitored. The arrow indicates the point of induction with 1 mM PPN.

cine nitrile. After 5 min conversion time, no amidase activity could be detected, and the obtained ee value (70%, (*S*)-phenylglycine amide) clearly belongs to the enantioselective property of the NHase. The apparent *E*-value, according to Chen et al.,[14] was 6.3. After 10 min, phenylglycine was also detected and the enantioselective conversion of phenylglycine nitrile was a 'mixed' result of the two kinetically different enzymes. After 30 min reaction time, the ee of the (*S*)-phenylglycine was >99%. After 240 min conversion time, the (*S*)-phenylglycine nitrile was nearly completely converted to the corresponding amide and even further hydrolyzed to the phenylglycine. The amidase also prefers the (*S*)-enantiomer. Therefore, after 1440 min (24 h) the (*S*)-phenylglycine amide was totally converted to the (*S*)-phenylglycine and the ee value of the (*R*)-phenylglycine amide was >99%. The high preference for the (*S*)-phenylglycine amide was also described from the strain *Pantoea endophytica* 26.2.2 by our group.[6]

The bioconversion of α-amino nitriles such as phenylglycine nitrile is disturbed by the spontaneous chemical degradation to aldehyde and ammonia via a retro-Strecker reaction.[15] This reaction takes place at pH value 7 which was optimal for the enzymatic reaction. The total loss of the balance in the experiment shown in Figure 2 amounted to 40%.

2.3. Conversion of (*RS*)-2-phenylpropionitrile

Permeabilized and lyophilized cells of *K. oxytoca* were incubated with (*RS*)-2-phenylpropionitrile, and the turnover was analyzed by chiral HPLC. 2-Phenylpropionitrile was almost stoichiometrically converted to 2-phenylpropionamide within 120 min (Fig. 3). The NHase was shown to generate preferentially the (*S*)-enantiomer of its product. The presence of an amidase was demonstrated by the subsequent conversion of 2-phenlypropionamide to 2-phenylpropionic acid. Additionally, a (*S*)-selectivity of the amidase was also revealed. After 1440 min (24 h) conversion time at 15 °C, the enantiomeric excess of the (*R*)-phenylpropionamide was 78% (Fig. 3). The impact of the amidase on the enantiomeric excess of the formed 2-phenylpropionamide could be neglected up to 60% conversion of 2-phenylpropionitrile. At this point, the concentration of the 2-phenylpropionic acid formed was less than 1.5% of the formed 2-phenylpropionamide concentration.

Bauer et al.[10] isolated *A. tumefaciens* d3 in a screening using 2-phenylpropionitrile as the sole source of nitrogen, where both

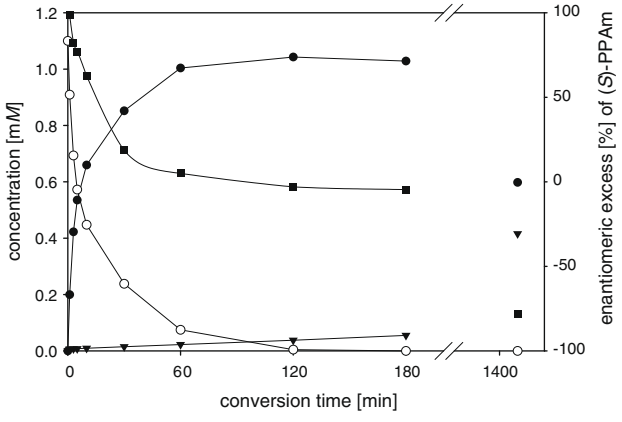

Figure 3. Conversion of (*RS*)-2-phenylpropionitrile by permeabilized and lyophilized cells of *Klebsiella oxytoca* 38.1.2 (15 °C, 40 mL-scale). The concentrations of 2-phenylpropionitrile (○); 2-phenylpropioamide (●) and 2-phenylpropionic acid (▼) were monitored. The ee value of (*S*)-2-phenylpropioamide (■) was calculated by comparison of the peak areas of the respective (*S*)- and (*R*)-enantiomers obtained by chiral HPLC.

the NHase and amidase preferentially converted the (*S*)-enantiomer of their respective substrate. Unlike *K. oxytoca* 38.1.2, the amidase of *A. tumefaciens* d3 showed higher activity as the NHase. 2-Phenylpropionamide was found only transiently in small amounts during the conversion of 2-phenylpropionitrile to 2-phenylpropionic acid. To investigate the NHase activity of *A. tumefaciens*, it was necessary to inhibit the amidase with diethyl phosphoramidate.[10]

Since the enantioselectivity of enzymatic processes may be influenced by the reaction conditions (e.g., temperature, pH, medium)[16] the conversion was performed at different temperatures (15–37 °C). An apparent *E*-value could be calculated according to Chen et al.[14] The apparent enantiomeric ratio near 50% conversion of 2-phenylpropionitrile rose from 16 to 35 with increasing temperature (Table 1). A pronounced increase of the NHase enantioselectivity of the whole-cell reaction at higher temperatures was also demonstrated for the conversion of ketoprofen nitrile by Bauer et al.[17] A further improvement of enantioselectivity was obtained after the purification of the NHase (ee values > 90 % until about 30 % conversion).[7,18]

Table 1
Influence of the temperature on the enantioselective hydrolysis of *rac*-phenylpropionitrile using permeabilized and lyophilized cells of *Klebsiella oxytoca* 38.1.2 (40 mL scale, pH 7.0)

Temperature (°C)	15	23	30	37
Conversion of PPN (%)	49	46	43	46
ee of (*S*)-PPAm[a] (%)	77	85	87	88
E_{app}[b]	16	26	28	35

The initial enzyme activity was 180 nkat.
[a] The enantiomeric excess (ee) was calculated by comparison of the peak areas obtained from chiral HPLC of the respective (*S*)- and (*R*)-enantiomer.
[b] The apparent *E*-value ($E_{app.}$) was calculated according to Chen et al.[14]

2.4. Conversion of (*RS*)-mandelonitrile

Permeabilized and lyophilized cells of strain 38.1.2 were incubated at different temperatures with *rac*-mandelonitrile, and the turnover was analyzed by chiral HPLC (the data are shown in Table 2). Due to the instability of mandelonitrile and reverse chemical reaction of benzaldehyde with cyanide in phosphate buffer to form *rac*-mandelonitrile,[19] a theoretically 100% yield of enantiopure mandelamide (MAm) is possible by asymmetric catalysis (the reaction scheme is shown in Fig. 4).

Analogously to the conversions of (*RS*)-phenylglycine nitrile and (*RS*)-2-phenylpropionitrile, *rac*-mandelonitrile was converted

Table 2
Influence of the temperature on the enantioselective conversion of *rac*-mandelonitrile by the NHase of *Klebsiella oxytoca* 38.1.2 using pretreated[a] permeabilized and lyophilized cells

Temperature (°C)	15	23	30	37
Yield[b] (MAm) after 4 h (%)	34	41	34	35
Yield[b] (MAm) after 24 h (%)	66	80	48	37
ee[c] of (*S*)-MAm (%)	91	95	89	94
Residual activity[d] after 4 h	n.d.	50%	50%	0
Residual activity[d] after 24 h	71%	17%	0	0

The initial activity was 57 nkat (NHase activity was assayed with PPN as substrate at 30 °C and pH 7).
n.d. = not determined.
[a] See Section 4 for details.
[b] The yield is based on the initial MN concentration in the reaction medium.
[c] The enantiomeric excess (ee) was calculated by comparison of the peak areas obtained from chiral HPLC of the respective (*S*)- and (*R*)-enantiomer.
[d] The residual activity was assayed with PPN as substrate at 30 °C and pH 7.

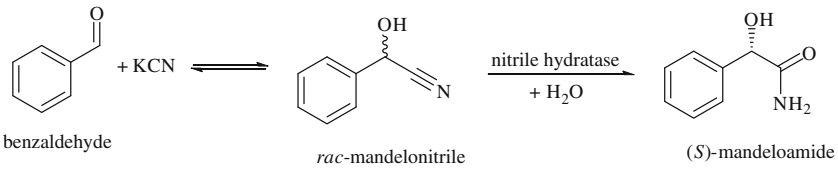

Figure 4. Reaction scheme for the biotransformation of *rac*-mandelonitrile to (*S*)-mandeloamide using permeabilized and lyophilized cells of *Klebsiella oxytoca* 38.1.2.

enantioselectively to the corresponding (*S*)-amide. As expected for asymmetric resolution, the observed enantiomeric excesses of the formed mandelamide remained almost constant during the conversion. At all temperatures, almost no amidase activity could be detected and the subsequent reaction of mandelamide to mandelic acid was less than 1% after 1440 min (24 h) conversion time. One side reaction of this conversion was the reduction of benzaldehyde to benzylalcohol, which is probably catalyzed by an NAD(P)H-dependent alcohol dehydrogenase. The suppression of the reaction should be arranged by incubating the permeabilized cells in a buffered benzaldehyde solution. Although the lyophilized cells were incubated with benzaldehyde prior to biotransformation, the reaction concerning the transformation of benzaldehyde to benzylalcohol was not completely suppressed (see Fig. 5) and rose with increasing temperatures (data not shown).

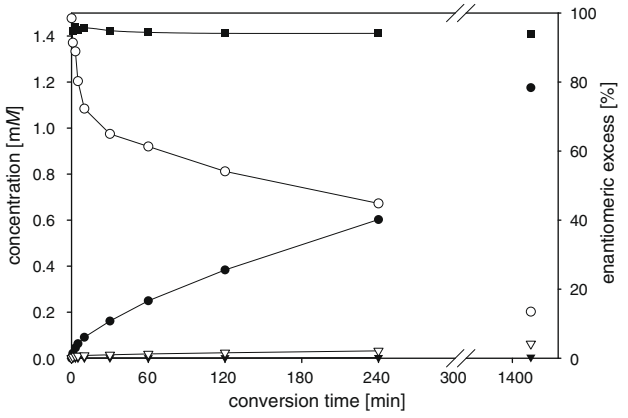

Figure 5. Conversion of (*RS*)-mandelonitrile by permeabilized and lyophilized cells of *Klebsiella oxytoca* 38.1.2 (23 °C, 40 mL-scale). Reaction conditions and pretreatment of the lyophilisate as reported in Section 4. The concentrations of mandelonitrile (○), mandelamide (●) mandelic acid (▼), benzylalcohol (▽) were monitored. The ee value of (*S*)-mandeloamide (■) was calculated by comparison of the peak areas of the respective (*S*)- and (*R*)-enantiomers obtained by chiral HPLC.

In general, NHases are sensitive to cyanohydrins, with aldehydes and hydrogen cyanide constituting the same species. As the enzyme activity was stable for 24 h at all tested temperatures when the lyophilisate was incubated in buffer (data not shown), the loss of activity during conversion is a consequence of the inhibiting resp. inactivating influence of the benzaldehyde present in the reaction mixture. The inhibition constants K_{Iapp} of benzaldehyde and cyanide at 30 °C were 0.1 mM and 0.03 mM, respectively. Although the measured initial activity was highest at 37 °C, the highest yield of mandelamide was obtained at lower temperatures, due to minor inactivation of the NHase activity from benzaldehyde and cyanide. The highest mandelamide yield of 80% with an enantiomeric excess of 95% for the (*S*)-enantiomer could be reached after 24 h conversion at 23 °C (Fig. 5).

Tamura[20] described a method for producing (*S*)-mandelamide by maintaining the cyanohydrin concentration at a definite level with the use of an automatic cyanohydrin controller. With this method, the inhibitory effect of benzaldehyde and cyanide could be reduced. It was possible to produce (*S*)-mandelamide from mandelonitrile up to a concentration of 1522 mM in 22 h with an enantiomeric purity of 95% at 98.7% conversion rate using *Rhodococcus* sp. HT40-6.

For the NHase of *K. oxytoca* 38.1.2, a stabilizing effect of the temperature was demonstrated. The residual activity after 24 h bioconversion at 23 °C was only 17%, whereas at 15 °C a residual activity of 71% could be determined after 24 h.

3. Conclusion

A gram-negative strain identified as *K. oxytoca* was isolated from an enrichment using 2-phenylpropionitrile as the sole source of nitrogen. The maximum NHase activity of the cultivation in a 550 mL scale was 3.5 μkat g^{-1} dry biomass.

By performing several biotransformations with α-arylnitriles it was shown that the isolated microorganism induced a (*S*)-enantioselective NHase and a putative (*S*)-selective amidase. During a time course study of the conversion of (*RS*)-phenylglycine nitrile, the NHase/amidase system resulted in the generation of both (*S*)-phenylglycine (ee >99%) and (*R*)-phenylglycine amide (ee >99%). The conversion of (*RS*)-2-phenylpropionitrile to (*S*)-phenylpropionamide resulted in an apparent enantiomeric ratio of 16–35 depending on the temperature. (*RS*)-Mandelonitrile was also enantioselectively converted to the (*S*)-amide. After 24 h conversion at 23 °C a mandelamide yield of 80% with an enantiomeric excess of 95% for the (*S*)-enantiomer was reached without further conversion to mandelic acid.

4. Experimental

4.1. Chemicals

All chemicals were of analytical grade purity and supplied from Fluka-Sigma–Aldrich (Taufkirchen), Merck (Darmstadt), ABCR (Karlsruhe). (*R*)-Mandeloamide, (*R*)- and (*S*)-phenylglycine nitrile, (*R*)- and (*S*)-phenylglycine amide as well as (*R*)-and (*S*)-phenylglycine were a gift from the department of Biocatalysis & Organic Chemistry, Delft University of Technology, The Netherlands.

4.2. Bacterial strain

Bacteria were isolated by enrichment cultivations with PPN as sole source of nitrogen as previously described.[6] The isolate 38.1.2 was initially classified according to a Gram, KOH and aminopeptidase test (Bactident aminopeptidase: Merck, Darmstadt, Germany). The strain was classified using 16S-rDNA sequence analysis. The almost 16S-rDNA was amplified by PCR and the resulting fragment was partially sequenced. The nucleotide sequence obtained was compared with the NCBI database using the program BLASTN.[21] The strain 38.1.2 was identified as *K. oxytoca*.

4.3. Cultivation conditions

For the cultivation of *K. oxytoca,* the following medium composition was used for shaking flask cultivations: 20 g glucose, 0.3 g

$(NH_4)_2SO_4$, 1.4 g KH_2PO_4, 7 g Na_2HPO_4, 0.02 g Fe(III)–citrate, 1 g $Mg_2SO_4 \cdot 7\ H_2O$, $CaCl_2 \cdot 2H_2O$, 1 mL trace element solution (70 mg L^{-1} $ZnCl_2$, 100 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 62 mg L^{-1} H_3BO_4, 190 mg L^{-1} $CoCl_2 \cdot 6H_2O$, 17 mg L^{-1} $CuCl_2 \cdot 2H_2O$, 24 mg L^{-1} $NiCl_2 \cdot 6H_2O$, 36 mg^{-1} L $Na_2MoO_4 \cdot 2H_2O$ and 1.3 mL L^{-1} HCl (25%)) and 3 mL vitamin solution.[22]

The strain 38.1.2 was cultivated overnight in 50 mL complex medium (yeast extract, tryptone, soy peptone with 2 g L^{-1} each) at 25 °C under shaking (100 rpm, amplitude 50 mm) in 300 mL-Erlenmeyer flask with baffles. The preculture was transferred to a 2 L-Erlenmeyer flask with baffles containing 500 mL minimal medium described above. The culture was incubated at 25 °C and 100 rpm (amplitude 50 mm). At 100% ammonia depletion, the NHase activity was induced by addition of PPN (1 mM final concentration). The cells were harvested 12 h after induction by centrifugation (13,000*g*, 30 min, 4 °C) and were washed twice with Na_2HPO_4/KH_2PO_4 buffer (50 mM, pH 7.0). Afterwards the cells were incubated for 30 min at 25 °C with 0.1% (w/v) Triton-X 100 in Na_2HPO_4/KH_2PO_4 buffer (50 mM, pH 7.0), centrifuged (13,000*g*, 30 min, 4 °C) and washed again. The permeabilized cells were lyophilized and stored at −20 °C.

4.4. Determination of enzyme activities

The cell suspension (1 mL) was centrifuged (16,000*g*, 5 min) and the cell pellet was washed once with Na_2HPO_4/KH_2PO_4 buffer (50 mM, pH 7.0). The cells were resuspended in 750 μL Na_2HPO_4/KH_2PO_4 buffer (50 mM, pH 7.0) and were incubated for 5 min at 30 °C in a thermomixer at 1000 rpm. The reaction was started by addition of 250 μL of 4 mM PPN in Na_2HPO_4/KH_2PO_4 buffer (50 mM, pH 7.0) to a final concentration of 1 mM. The reaction was stopped after 1 min by addition of 100 μL 1 M HCl. The cells were removed by centrifugation (16,000*g*, 5 min), and the formation of the amide was analyzed by HPLC. One μkat of NHase activity is defined as the amount of enzyme that catalyzed the formation of 1 μmol PPA per second.

4.5. Determination of K_{Mapp} and K_{Iapp}

Kinetic parameters were determined in 1 mL scale from the initial reaction rates of 0.25–2 mM PPN in Na_2HPO_4/KH_2PO_4 buffer (50 mM, pH 7.0). Enzyme and substrate solutions were preincubated at 30 °C separately. The reaction was started by adding enzyme suspension (2.5 nkat) to substrate solution. The reaction was stopped after 1 min by adding 100 μL 1 M HCl. The formation of PPA was analyzed by HPLC. K_{Mapp} were calculated according to the linearization method of Hanes.

For the determination of K_I values of benzaldehyde and cyanide, the IC 50 values were determined. Therefore, the initial reaction rates were measured with 1 mM PPN supplemented with benzaldehyde resp. potassium cyanide in a concentration range from 0.25 to 5 mM. The reactions were carried out and analyzed as described above. Using the GraphPad Prism 4 software, IC_{50} were calculated using nonlinear regression. The apparent K_I values for benzaldehyde and cyanide were calculated according to Cheng and Prusoff.[23]

4.6. Biotransformation

For the conversion of mandelonitrile, 100 mg permeabilized and lyophilized cells were incubated overnight with 50 mL 5 mM benzaldehyde in Na_2HPO_4/KH_2PO_4 buffer (50 mM, pH 7.0) at 25 °C and were washed two times with Na_2HPO_4/KH_2PO_4 buffer (50 mM, pH 7.0) prior use in order to reduce the reactions concerning alcohol- and aldehyde-dehydrogenase activity. For the conversion of 2-phenylpropionitrile, 100 mg lyophilized cells were directly resuspended in Na_2HPO_4/KH_2PO_4 buffer (50 mM, pH 7.0).

The biotransformations of *rac*-phenylglycine nitrile, *rac*-phenylpropionitrile and *rac*-mandelonitrile were performed on a 40 mL-scale. The cell suspensions were incubated for 10 min at the respective temperature. The reaction was started by addition of the nitrile to a final concentration of 1 mM. Samples (1 mL) were periodically withdrawn and added to 100 μL of 1 M HCl to stop the reaction. The cells were removed by centrifugation (16,000*g*, 5 min) and the conversion products were analyzed by HPLC.

4.7. Analytical methods

The glucose concentration of the cultivation broth was measured using the Granu test kit (Merck, Darmstadt). The ammonia concentration was determined using the Spectroquant ammonium test kit (Merck, Darmstadt).

4.8. HPLC analysis

Nitriles and the bioconversion products were analyzed by a Thermo Finnigan Surveyor HPLC System equipped with a 4 channel pump, autosampler and UV/VIS detector at 210 nm. A Grom-Sil 120 ODS-3cp 3 μm (125 × 4.6 mm) reverse phase column at a flow of 0.7 mL min^{-1} was used. The used mobile phase was acetonitrile and 0.3% (v/v) H_3PO_4 in a ratio of 50:50 (v/v) (phenylpropionitrile and conversion products) and 20:80 (v/v) (mandelonitrile and conversion products), respectively.

The conversion of (*RS*)-phenylglycine nitrile to phenylglycine amide (*R* and *S*) and phenylglycine (*R* and *S*) was determined by chiral HPLC on a Daicel Chemical Industries Ltd, 150 × 4.6 mm 5 μ Crownpak CR (+) column. The eluent was aqueous $HClO_4$, pH 1.3 at a flow rate of 0.6 mL min^{-1}, the column temperature was 16 °C. The enantiomers were detected at a wavelength of 215 nm.

For separation of the enantiomers of 2-phenylpropionamide, the samples were lyophilized and dissolved afterwards in half of the previous volume *n*-hexane: isopropanol (80:20, v/v). The samples were analyzed using a Chiracel OD 4.6 × 250 mm column (Daicel Chemical Industries Ltd) on a Spectra System (AS3000, P2000, UV2000 at 210 nm) from Thermo Separation Products. As mobile phase *n*-hexane/isopropanol (80:20, v/v) was used at a flow rate of 0.5 mL min^{-1}.

For separation of the enantiomers of mandelamide, a Nucleodex β-OH column (Macherey & Nagel) was used. As mobile phase 50% (v/v) $MeOH/H_2O_{bidest.}$ (50:50, v/v) was used at a flow rate of 0.7 mL min^{-1}. The enantiomers were detected at a wavelength of 215 nm. For the analysis of the enantiomers of mandelic acid, the samples were lyophilized and dissolved afterwards in half of the previous volume *n*-hexane/isopropanol/trifluoroacetic acid (80:20:1, v/v/v). The enantiomers of mandelic acid were separated by using a Chiracel OD, 250 × 4.6 mm column (Daicel Chemical Industries Ltd). The mobile phase was *n*-hexane/isopropanol/trifluoroacetic acid (80:20:1, v/v/v) at a flow rate of 0.5 mL min^{-1}. The enantiomers were detected at a wavelength of 210 nm.

The enantiomeric excess was calculated by comparison of the peak areas obtained from chiral HPLC of the respective (*S*)- and (*R*)-enantiomer. The enantiomeric ratio (*E*) of the conversion of PPN was calculated according to Chen et al.[14]

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