DOI: 10.1002/adsc.200900087

Construction of Recombinant *Escherichia coli* Catalysts which Simultaneously Express an (*S*)-Oxynitrilase and Different Nitrilase Variants for the Synthesis of (*S*)-Mandelic Acid and (*S*)-Mandelic Amide from Benzaldehyde and Cyanide

Olga Sosedov,[a] Kathrin Matzer,[a] Sibylle Bürger,[a] Christoph Kiziak,[a,b] Stefanie Baum,[a] Josef Altenbuchner,[c] Andrzej Chmura,[d] Fred van Rantwijk,[d] and Andreas Stolz[a,*]

[a] Institut für Mikrobiologie, Universität Stuttgart, Allmandring 31, 70569 Stuttgart, Germany
Fax: (+49)-711-685-65725; e-mail: Andreas.Stolz@imb.Uni-Stuttgart.de
[b] Present address: Lonza AG, Biotechnology Research and Development, 3930 Visp, Switzerland
[c] Institut für Industrielle Genetik, Universität Stuttgart, Allmandring 31, 70569 Stuttgart, Germany
[d] Laboratory of Biocatalysis and Organic Chemistry, Department of Biotechnology, Delft University of Technology, Julianalaan 136, 2628 BL Delft, The Netherlands

Received: February 10, 2009; Revised: May 15, 2009; Published online: June 16, 2009

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.200900087.

Abstract: Recombinant *Escherichia coli* strains were constructed which simultaneously expressed the genes encoding the (*S*)-oxynitrilase from cassava (*Manihot esculenta*) together with the wild-type or a mutant variant of the arylacetonitrilase from *Pseudomonas fluorescens* EBC191 in a single organism under the control of a rhamnose-inducible promoter. The whole cell catalysts obtained converted benzaldehyde and potassium cyanide in aqueous media at pH 5.2 mainly to (*S*)-mandelic acid and/or (*S*)-mandelic amide and synthesized only low amounts of the corresponding (*R*)-enantiomers. The conversion of benzaldehyde and potassium cyanide (KCN) by a whole-cell catalyst simultaneously expressing the (*S*)-oxynitrilase and the wild-type nitrilase resulted in a ratio of (*S*)-mandelic acid to (*S*)-mandelic amide of about 4:3. This could be explained by the strong nitrile hydratase activity of the wild-type nitrilase with (*S*)-mandelonitrile as substrate. The relative proportion of (*S*)-mandelic amide formed in this system was significantly increased by coexpressing the (*S*)-oxynitrilase with a carboxy-terminally truncated variant of the nitrilase. This whole-cell catalyst converted benzaldehyde and KCN to mandelic amide and mandelic acid in a ratio of about 9:1. The *ee* of the (*S*)-mandelic amide formed was calculated to be $>95\%$.

Keywords: amide formation; cascade reactions; enantioselectivity; hydroxynitrile lyase; nitrilase

Introduction

Nitriles are used as intermediates in a wide range of synthetic reactions in the chemical industry and there is a considerable interest to use microorganisms or enzymes to synthesize or convert organic nitriles. These biotransformations allow chemo-, stereo- or enantioselective reactions that are often difficult to perform in synthetic chemistry and are usually more benign to the environment than traditional chemical processes.[1] An interesting group of nitriles for chiral synthetic processes are α-hydroxy nitriles, because these compounds can be easily chemically prepared and further converted to α-hydroxy amides and α-hydroxy carboxylic acids which are important products in the chemical industry.[2] There are two main enzymatic pathways known which result in the formation of optical active 2-hydroxy acids from α-hydroxy nitriles (Figure 1).

The most versatile type of reactions uses oxynitrilases from plants which catalyse the enantioselective addition of HCN to aldehydes or ketones.[3,4] Different oxynitrilases produce either (*S*)- or (*R*)-α-hydroxy nitriles often with a high degree of enantioselectivity; these subsequently can be chemically or enzymatically converted in enantioconservative reaction steps to

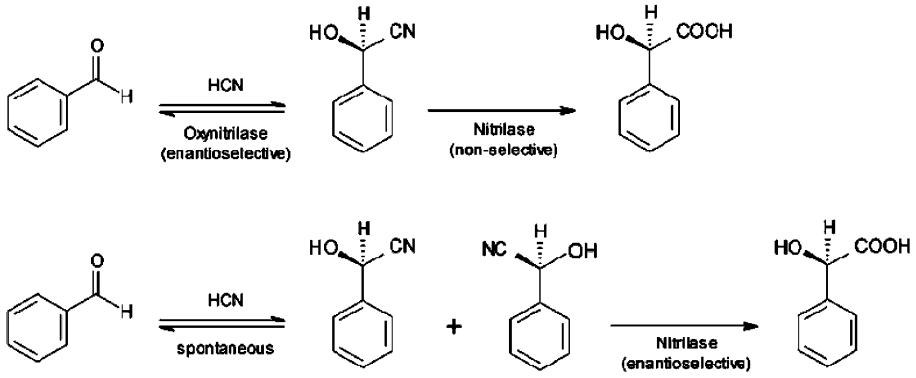

Figure 1. Schematic representation of the possible pathways for the synthesis of optical active mandelic acid using nitrilases and oxynitrilases

chiral α-hydroxy acids.[5,6] The second established biotransformation process applies the (reversible) chemical addition of HCN to aldehydes and ketones (resulting in racemic hydroxy nitriles) combined with enantioselective nitrilases. Unfortunately, only very few truly enantiospecific nitrilases are currently known and these nitrilases have only been shown to convert (substituted) mandelonitrile(s) to (*R*)-mandelic acid(s).[7–11]

We are currently attempting to combine enantiospecific oxynitrilases and bacterial nitrilases in a "one-pot" reaction in order to synthesize enantiopure α-hydroxy carboxylic acids. In practice, this process is hampered because both reactions must be performed using rather different conditions. Thus, synthetic oxynitrilase reactions are usually performed at pH <5 in order to suppress the uncatalyzed (and therefore non-enantiospecific) hydrocyanation reactions. Furthermore, the synthetic oxynitrilase reactions are usually performed in the presence of large amounts of organic solvents in order to further suppress the reversible chemical reaction and to provide a reservoir of HCN.[12] In contrast, the currently known nitrilases possess their pH optima close to pH 7 and are usually inactivated at higher concentrations of organic solvents.[13,14]

We have used two different strategies in order to combine the two enzymes for synthetic purposes. First, methods were established to combine the enzymes under *in vitro* conditions as (immobilized) biocatalysts. Thus, purified preparations of an (*S*)-specific oxynitrilase from cassava (*Manihot esculenta*) were co-immobilized with an arylacetonitrilase from *Pseudomonas fluorescens* EBC191, which converts a broad range of α-substituted nitriles and it was demonstrated that bienzymatic cross-linked enzyme aggregates (CLEAs) could be generated which converted benzaldehyde and HCN to (*S*)-mandelic acid.[16] The second strategy focuses on the construction of acid-tolerant whole-cell catalysts. We have previously described the construction of a recombinant derivative of the methylotrophic yeast *Pichia pastoris*, which simultaneously expressed the genes encoding the (*S*)-specific oxynitrilase from cassava and the arylacetonitrilase from *P. fluorescens* EBC191 under the control of individual *AOX1* promotors. This whole-cell catalyst converted benzaldehyde plus cyanide in an aqueous medium at a pH of 3.8 to mandelic acid and mandelic amide with a large surplus of the corresponding (*S*)-enantiomers.[17] Unfortunalely, only rather low enzyme activities were observed and still some of the corresponding (*R*)-enantiomers were formed. In order to solve these problems, it was attempted in the present study to combine both enzyme activities in a recombinant *E. coli* strain in order to generate "bienzymatic whole-cell catalysts" with higher specific activities for the desired reaction.

Results

Construction of a Recombinant *E. coli* Strain Simultaneously Carrying an Oxynitrilase and a Nitrilase Gene

Plasmid pIK9 was used to express the nitrilase gene from *Pseudomonas fluorescens* EBC191. (For the plasmids used and constructed in this study see Table 1.) This expression plasmid is a pBR322-derivative carrying an ampicillin resistance gene and allows the highly efficient expression of the cloned nitrilase under the control of a rhamnose-inducible promoter.[15]

Plasmid pQE4-MeHNL was used as source of the hydroxynitrile lyase gene from cassava (*Manihot esculenta*).[18] The hydroxynitrile lyase gene was amplified by PCR and subsequently transferred into the expression vector pAW229 giving plasmid pJOE 5361.1 as described in the Experimental Section. Plasmid pAW229 is a pACYC184 derivative carrying a chloramphenicol resistance gene and the $rhaP_{BAD}$ promoter.[19] Therefore, in plasmid pJOE 5361.1 the hydroxy-

Table 1. Plasmids used or constructed in the present study.

Plasmid	Relevant characteristics	Source or ref.
pAW229	Cam^r	[19]
pIK9	Plasmid pJOE2775 with the nitrilase gene from *P. fluorescens* EBC 191 carrying a C-terminal $(His)_6$-tag	[15]
pCK105	C-terminally deleted nitrilase (DelC-60) in pJOE2702	[23]
pQE4-MeHNLwt	Gene encoding the oxynitrilase from *Manihot esculenta* cloned into plasmid pQE4	[18]
pJOE5356.1	Part of the gene encoding the oxynitrilase from *Manihot esculenta* cloned into plasmid pAW229	[17]
pJOE5361.1	Complete gene encoding the oxynitrilase from *Manihot esculenta* cloned into plasmid pAW229	[17]

nitrile lyase was under the control of the same rhamnose-inducible promoter as present in plasmid pIK9. Thus it was possible to simultaneously express both genes in a single strain by the addition of rhamnose.

Simultaneous Expression of the Cassava Oxynitrilase and the *Pseudomonas fluorescens* EBC191 Nitrilase in *Escherichia coli* JM109(pIK9)(pJOE5361.1)

E. coli JM109(pIK9) was transformed with plasmid pJOE5361.1 and putative transformants carrying both plasmids were selected on LB-medium with ampicillin ($100\ \mu g\,mL^{-1}$) plus chloramphenicol ($20\ \mu g\,mL^{-1}$). The obtained clones of *E. coli* JM109(pIK9)(pJOE5361.1) were grown in liquid culture on the selective medium and the nitrilase and oxynitrilase genes induced by the addition of rhamnose (0.2% w/v). For comparison, also *E. coli* JM109(pIK9) and *E. coli* JM109(pJOE5361.1) were analyzed in the same way. The cells were harvested after 1–18 h incubation with rhamnose and protein synthesis analyzed by SDS-PAGE. These experiments demonstrated that *E. coli* JM109(pJOE5361.1) and *E. coli* JM109(pIK9)(pJOE5361.1) indeed synthesized a protein with the size of the cassava oxynitrilase. Furthermore, the experiments suggested that the oxynitrilase expression was more pronounced in *E. coli* JM109(pJOE5361.1) than in *E. coli* JM109(pIK9)(pJOE5361.1) and that in *E. coli* JM109(pIK9)(pJOE5361.1) the nitrilase was much more efficiently expressed than the oxynitrilase. Furthermore, it was found that both proteins were optimally synthesized after prolonged incubation times (>12 h) of the recombinant strains with rhamnose. SDS-PAGE gel electrophoresis suggested that the oxynitrilase and nitrilase proteins represented about 2–3% and 5%, respectively, of the totally formed soluble proteins (see Figure S1 in the Supporting Information).

Detection of Oxynitrilase and Nitrilase Activities in *E. coli* JM109(pIK9)(pJOE5361.1)

The enzymatic formation and turn-over of mandelonitrile had to be analyzed at pH $\leq$ 5.5 in order to suppress the spontaneous (non-enantioselective) chemical formation or decomposition of mandelonitrile.[6] *E. coli* JM109(pIK9)(pJOE5361.1) was grown overnight in LB-medium plus ampicillin ($100\ \mu g\,mL^{-1}$), chloramphenicol ($20\ \mu g\,mL^{-1}$) and rhamnose (0.2% w/v). The cells were harvested by centrifugation, resuspended in sodium citrate buffer (25 mM, pH 5.2) and the nitrilase activity determined with resting cells and mandelonitrile (10 mM) as substrate using HPLC. These cells converted mandelonitrile to mandelic acid and mandelic amide in a ratio of approximately 4:1 as previously also shown for *E. coli* JM109(pIK9).[15] For *E. coli* JM109(pIK9)(pJOE5361.1) a nitrilase specific activity of 2.5 U/mg of protein was calculated. (In contrast, the wild-type cells of *Pseudomonas fluorescens* EBC191 demonstrated about 0.7 U/mg with mandelonitrile as substrate.[24])

The oxynitrilase activity of the whole cells was estimated by incubating the cells in 50 mM sodium citrate buffer (pH 5) with benzaldehyde (5 mM) and KCN (5 mM) and analyzing the reactions by reversed-phase chromatography. The analysis suggested that *E. coli* JM109(pIK9)(pJOE5361.1) indeed converted benzaldehyde faster than observed in control experiments with cells of *E. coli* JM109(pIK9) which only synthesized the nitrilase activity. For the oxynitrilase a specific activity of about 0.4 U/mg of protein was estimated from the decrease in the benzaldehyde concentration (corrected for the rate of the spontaneous decrease in the benzaldehyde concentration). The cells of *E. coli* JM109(pIK9)(pJOE5361.1) synthesized in this experiment mandelic acid and mandelic amide in a ratio of 1.2:1. In contrast, *E. coli* JM109(pIK9) produced from racemic mandelonitrile only about 19% amide.[15] This indicated that this strain indeed formed a large proportion of (*S*)-mandelonitrile because it was earlier found that the nitrilase from *P. fluorescens* EBC191 formed from (*S*)-mandelonitrile approximately the same amounts of (*S*)-mandelic amide and (*S*)-mandelic acid, whereas (*R*)-mandelonitrile was converted to (*R*)-mandelic acid and (*R*)-mandelic amide in a ratio of about 9:1.[15,16]

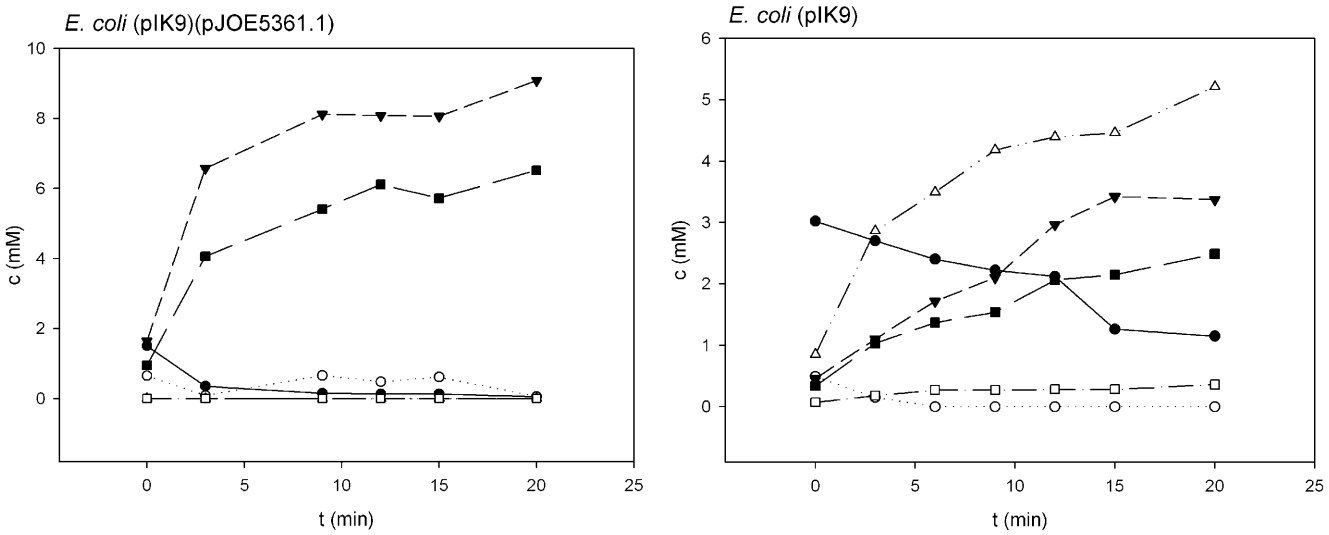

Figure 2. Conversion of benzaldehyde and KCN by resting cells of *E. coli* JM109(pIK9)(pJOE5361.1) (*left*) and *E. coli* JM109(pIK9) (*right*). The reaction mixtures contained in a total volume of 2 mL 100 mM sodium citrate buffer (pH 5.0), 15 mM benzaldehyde and resting cells ($OD_{546nm}=20$). The reactions were started by the addition of KCN (15 mM). Aliquots (200 μL each) were taken at the indicated time intervals and the reactions terminated by the addition of 20 μL 1 M HCl. The cells were removed by centrifugation in an Eppendorf centrifuge (5 min, 14,000 *g*). The concentrations of benzaldehyde (●), (*R*,*S*)-mandelonitrile (○), (*R*)-mandelic amide (□), (*S*)-mandelic amide (■), (*R*)-mandelic acid (△), and (*S*)-mandelic acid (▼) were determined in the supernatants by reversed phase and chiral HPLC.

Enantioselective Formation of (*S*)-Mandelic Acid from Benzaldehyde and KCN using *E. coli* JM109(pIK9)(pJOE5361.1)

The simultaneous production of the cassava oxynitrilase and the *Pseudomonas* nitrilase in one recombinant organism should allow the enantioselective synthesis of (*S*)-mandelic acid from benzaldehyde and cyanide, because the cassava oxynitrilase is able to catalyze the highly enantiospecific conversion of benzaldehyde and cyanide to (*S*)-mandelonitrile[20] and the nitrilase from *P. fluorescens* EBC191 is able to convert (*S*)-mandelonitrile to (*S*)-mandelic acid.[6,15] In the following experiment it was tested if it was possible to completely suppress the formation of (*R*)-mandelic amide and (*R*)-mandelic acid using the recombinant *E. coli* strain. Therefore, the amount of resting cells was increased and the reaction times decreased compared to the previously described experiment. The reactions were analyzed by reversed-phase and chiral HPLC and it was found that it was indeed possible to obtain only the (*S*)-enantiomers of the amide and the acid (Figure 2, *left*; Table 2 entry A). In contrast, in the control experiment with *E. coli* JM109-(pIK9) more (*R*)-mandelic acid than (*S*)-mandelic acid was formed (Figure 2, *right*).

Optimization of the Reaction Conditions

The previously described reactions were performed with rather low equimolar concentrations of benzaldehyde and KCN (5–15 mM). In the following experiments increasing initial benzaldehyde concentrations

Table 2. Whole cell conversions of benzaldehyde and cyanide using *E. coli* JM109(pIK9) (pJOE5361.1).

Entry	Benzaldehyde [mM]	Cyanide [mM]	Phases[a]	Buffer	Time [h]	Conversion [%]	Acid/Amide	*ee* [%] (*S*)-acid
A	15	15	1	Citrate (pH 5, 100 mM)	0.33	98.0	1.4	>99
B	10	30	1	Citrate (pH 5, 200 mM)	0.5	96.1	1.3	>99
C	25	75	1	Citrate (pH 5, 200 mM)	0.5	95.0	1.2	>99
D	10	30	2	Citrate (pH 5, 50 mM)	7.5	84.2	1.7	>99
E	25	75	2	Citrate (pH 5, 50 mM)	7.5	96.6	1.6	>99
F	50	150	2	Citrate (pH 5, 50 mM)	7.5	99.0	1.4	>99
G	100	300	2	Citrate (pH 5, 50 mM)	7.5	96.4	1.4	>99

[a] The two-phase systems consisted of 70% (v/v) DIPE and 30% (v/v) 50 mM soduum citrate buffer (pH 5).

of 10–200 mM combined with the respective threefold concentrations of KCN were tested (in 250 mM sodium citrate buffer, pH 5.0). The reaction mixtures contained cells of *E. coli* JM109(pIK9)(pJOE5361.1) with an optical density (OD_{546nm}) of 2.6 and the reactions were started by the addition of benzaldehyde. The addition of increasing concentrations of KCN resulted in a significant alkalization of the media and the media containing 50–200 mM KCN had initial pH values of pH 9–13. Nevertheless, it was found that the whole cell catalysts were able to convert up to 50 mM benzaldehyde within 10 min and that even an initial benzaldehyde concentration of 100 mM was almost completely converted within 30 min. In contrast, the reaction mixtures containing 200 mM benzaldehyde were active only for about 10 min and only about 25% of the initially added benzaldehyde was converted. The termination of the reaction was correlated to an increased viscosity of the reaction mixtures which was probably caused by a rapid cell lysis at the alkaline pH value of the medium.

The *ee* values of the products formed [(*S*)-mandelic acid and (*S*)-mandelic amide] were >99% in the reactions with initial benzaldehyde concentrations of <50 mM (Table 2, entries B, C). The *ee* values significantly dropped (<50%) in those experiments with increasing substrate concentrations – very probably due to the increased pH values of the reaction media.

Oxynitrilases are usually applied *in vitro* in the "traditional" chemical synthesis in the presence of large amounts of organic solvents.[21,22] Therefore, the activity of the whole cell catalyst was also tested in aqueous-organic solvent two-phase systems consisting of 50 mM sodium citrate buffer (pH 5) and diisopropylether (DIPE) (70%). The cells were suspended in the aqueous phase to an optical density (OD_{546nm}) of 1.8 and cyanide was added to the organic phase from an HCN stock solution in DIPE. The reactions were started by the addition of 25–200 mM benzaldehyde (from a 2 M stock solution in DIPE). The observed reaction rates were significantly slower than those observed in the purely aqueous systems. Nevertheless, all reactions were completed in less than 7.5 h. The enantiopurity of the (*S*)-mandelic acid was in all reactions >99% (Table 2, entries D–F).

Increased Formation of Mandelic Amide by using a C-Terminally Truncated Nitrilase Variant

The nitrilase from *P. fluorescens* EBC191 formed from (*S*)-mandelonitrile approximately the same amounts of (*S*)-mandelic acid and (*S*)-mandelic amide.[6,16] Recently, several mutants of the nitrilase from *P. fluorescens* EBC191 have been generated and analyzed for amide formation and enantioselectivity and it was found that C-terminally truncated mutants

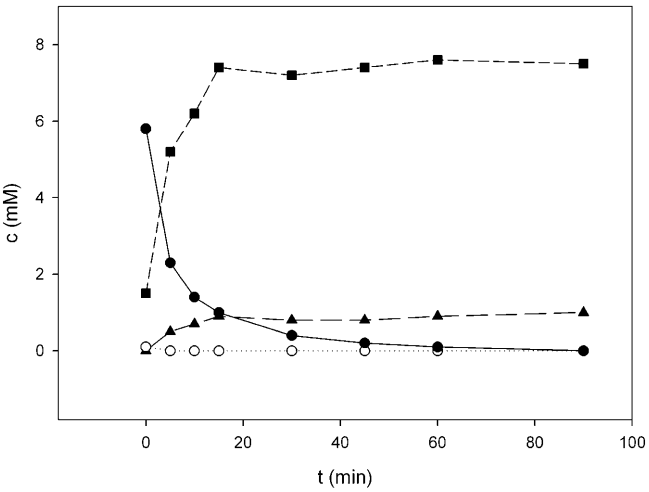

Figure 3. Conversion of benzaldehyde and KCN by resting cells of *E. coli* JM109(pCK105)(pJOE5361.1). The reaction mixtures contained in a total volume of 1 mL 100 mM sodium citrate buffer (pH 5.0), 10 mM benzaldehyde, 10 mM KCN and resting cells ($OD_{600nm}=22$). Aliquots (90 μL each) were taken at the indicated time intervals and the reactions terminated by the addition of 10 μL 1 M HCl. The cells were removed by centrifugation in an Eppendorf centrifuge (5 min, 14,000 *g*) and the concentrations of benzaldehyde (●), mandelic amide (■), mandelic acid (▲), and mandelonitrile (○) in the supernatants were analyzed by reversed phase HPLC.

of the nitrilase formed up to 50% of mandelic amide from racemic mandelonitrile.[23] It was therefore attempted to increase the amount of (*S*)-mandelic amide formed from benzaldehyde and KCN by co-expressing the (*S*)-oxynitrilase with the C-terminally deleted nitrilase variant (Del-C60) encoded on plasmid pCK105.[23] The corresponding whole-cell catalyst *E. coli* JM109(pCK105)(pJOE5361.1) indeed converted benzaldehyde plus cyanide to mandelic amide and mandelic acid in a ratio of about 9:1 (Figure 3). The analysis of a sample taken after 45 min by chiral HPLC suggested that only (*S*)-mandelic amide was present and no indications for (*R*)-mandelic amide were found. In order to analyze the accuracy of measurement, various defined amounts of (*R*)-mandelic amide were added to the samples. These experiments demonstrated that (*S*)-mandelic amide was formed with >95% *ee*.

Discussion

The results of the present study demonstrated that it is possible to simultaneously express a plant-derived oxynitrilase and a bacterial nitrilase in *E. coli* and that these recombinant "bienzymatic whole-cell catalysts" are efficient for the synthesis of (*S*)-mandelic acid and (*S*)-mandelic amide. The enantioselectivity

of the studied reactions presumably strongly depends on the relative proportions of the oxynitrilase and nitrilase (or nitrile hydratase) activities which are present in the respective biocatalysts. It is expected that the highly enantioselective formation of (*S*)-mandelic acid and/or (*S*)-mandelic amide will require a surplus of nitrilase activity in comparison to the oxynitrilase activities. This should enable the immediate and quantitative conversion of the enzymatically formed (*S*)-mandelonitrile by the nitrilase and result in the irreversible fixation of the stereochemistry at the relevant carbon atom, because the chemical racemization of mandelonitrile is suppressed. Obviously, this was achieved in the present study, because in all experiments only marginal amounts of mandelonitrile were observed. This indicated that in these cells the nitrilase activity was much higher than the oxynitrilase activity. This was confirmed by the comparison of the reaction rates of the whole cell catalysts with benzaldehyde plus cyanide or mandelonitrile, which also demonstrated significantly higher nitrilase activities than oxynitrilase activities. The comparably lower expression of the oxynitrilase activity could have been caused either by problems in the transcription/translation of the plant-derived oxynitrilase gene or by the fact that the oxynitrilase gene was cloned on a pACYC184-derived plasmid which presumably has a slightly lower copy number in LB-grown cells of *E. coli* compared to the pBR322-derived plasmid carrying the nitrilase gene.[25]

The recombinant *E. coli* clones that were generated in the present study appear to be better suited for the intended biotransformation than the previously constructed recombinant clones of *Pichia pastoris* although they are less acid-tolerant.[17] This was mainly due to the higher specific activities that were obtained for the *E. coli* strains compared to the derivatives of *P. pastoris*. Thus, it was found that the nitrilase activities were more than 7-times higher and the oxynitrilase activities more than 30-times higher in the recombinant *E. coli* strains than in the *Pichia* clones. This is presumably caused by the much higher copy number of the plasmid encoded nitrilase and oxynitrilase genes in the *E. coli* strains.

The wild-type nitrilase converted (*S*)-mandelonitrile to almost equimolar concentrations of (*S*)-mandelic acid and (*S*)-mandelic amide. This resulted in combination with the availability of mutant variants with an increased tendency to convert mandelonitrile to mandelic amide in the surprising observation that a combination of an oxynitrilase and a nitrilase could be used for the enantioselective synthesis of (*S*)-mandelic amide. This might open up a more general way for the enantioselective synthesis of alpha-substituted amides, which is currently hampered by the generally observed limited enantioselectivity of nitrile hydratases.[25]

The bienzymatic process demonstrated in the present study should have a wide range of possible applications because both enzymes, the (*S*)-oxynitrilase from cassava as well as the nitrilase from *P. fluorescens* EBC191, convert a wide range of aliphatic and aromatic substrates. Furthermore, for both enzymes already strategies for genetic modifications have been described which, in principle, allow further improvements with respect to substrate specifity, activity, and enantioselectivity.[23,27] We are currently attempting to obtain nitrilase mutants which either form quantitatively (*S*)-mandelic amide or (*S*)-mandelic acid from (*S*)-mandelonitrile in order to construct "bienzymatic catalysts" which convert benzaldehyde and cyanide to stoichiometric amounts of (*S*)-mandelic amide or (*S*)-mandelic acid.

Conclusions

We have succeeded in developing an efficient recombinant whole-cell catalyst in which a plant-derived and a bacterial enzyme were combined in order to perform a commercially relevant biotransformation reaction. We have shown that the double clone cells are efficient catalysts for synthesizing (*S*)-mandelic acid and (*S*)-mandelic amide. The catalytic efficiency of the double clone is better in aqueous buffer than in an aqueous-organic biphasic system but, in the former, pH control is rapidly lost and the resulting pH shift towards alkaline conditions reduces the applicability of the bienzymatic procedure. The biphasic system, in contrast, although slower, is more suitable in reactions using high concentrations of reactants. The utilization of nitrilase variants which produce large amounts of amides might open up a more general way for the enantioselective synthesis of α-substituted amides, which is currently hampered by the generally observed limited enantioselectivity of nitrile hydratases. We expect that this and similar bienzymatic cascades will be promising catalysts for the synthesis of enantiopure α-hydroxy carboxylic acids and α-hydroxy carboxylic amides.

Experimental Section

Chemicals

The chemicals used were obtained from Fluka (Neu-Ulm, Germany), Lancaster (Mühlheim/Main, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg), Bayer (Leverkusen, Germany), and Sigma–Aldrich (München, Germany). Restriction enzymes and other reagents for molecular biology were supplied by Roche Diagnostics (Mannheim, Germany) and NEB (Frankfurt/Main, Germany).

Bacterial Strains, Plasmids, and Culture Conditions

Escherichia coli JM 109 was used for all cloning experiments. The strain was routinely cultured in Luria-Bertani (LB) medium (plus 100 μg mL^{-1} ampicillin and/or 20 μg mL^{-1} chloramphenicol). Plasmid pIK9 has been described before.[15] *E. coli* M15(pQE4-MeHNLwt)[18] was kindly provided by Prof. H. Wajant (Würzburg, Germany).

DNA Manipulation Techniques

Digestion of DNA with restriction endonucleases (MBI Fermentas, St. Leon-Rot, Germany), electrophoresis, and ligation with T4 DNA ligase (MBI Fermentas) were performed according to standard procedures.[28] Transformation of *E. coli* was done by the method of Chung et al.[29]

Cloning of the Oxynitrilase Gene from *Manihot esculenta* in *E. coli*

The gene encoding the oxynitrilase from *Manihot esculenta* (MeHNL) was amplified by PCR from plasmid pQE4-MeHNLwt[18] using the primer pair s4299 (AAAAAACATATGG TAACTGCACATTTTGTT) and s4300 (AAAAAAAGCTTAAGCA TATGCATCAGCCAC). The PCR product was subsequently cut with *Nde*I and *Hind*III and the DNA fragment obtained ligated into the vector plasmid pAW229[19] which was previously cut with the same restriction enzymes. This resulted in the formation of plasmid pJOE5356.1 which contained the 5′-part of the gene encoding the MeHNL (because there was a *Hind*III-cleavage site within the PCR product). Subsequently, the PCR product obtained using the primer pair s4299 and s4300 and plasmid pJOE5356.1 were cleaved with *Hind*III. The insertion of the *Hind*III-fragment obtained from the PCR-product into the *Hind*III-cut plasmid pJOE5356.1 resulted in the formation of plasmid pJOE5361 which contained the complete MeHNL gene under the control of a rhamnose-inducible promoter.

Expression of the Oxynitrilase and Nitrilase in *E. coli*

For the induction of the enzymes in the recombinant *E. coli* strains, precultures (10 mL each) were grown in LB-medium with ampicillin (100 μg mL^{-1}) or ampicillin (100 μg mL^{-1}) plus chloramphenicol (20 μg mL^{-1}). From these precultures Erlenmeyer flasks (100–500 mL) with the same composition were inoculated to an initial optical density (OD_{546nm}) of about 0.02. The cultures were cultivated at 30 °C on a orbital shaker (100 rpm) until they reached an optical density of about 0.3. Then, 0.2% (w/v) L-rhamnose was added and the bacterial cultures further incubated under the same conditions as before. The cells were usually harvested about 12 h later.

Preparation of Cell-Free Extracts

The bacterial cultures were harvested by centrifugation (9500 *g*, 15 min, 4 °C), washed and resuspended in Na/K phosphate buffer (50 mM, pH 7.5) or Tris/HCl (50 mM, pH 7.5), respectively. The cells were disrupted by using a French Press (Aminco, Silver Springs, Md. USA) at 80 MPa. Cell debris was removed by centrifugation at 100,000 *g* for 60 min at 4 °C. Protein was determined by the method of Bradford[30] using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli[31] Gels were routinely stained with Coomassie blue. A molecular weight standard (Kaleidoskop, Bio-Rad, Richmond, CA, USA) and a commercially available sample of the oxynitrilase from *M. esculenta* (Fluka, Buchs, Switzerland) were used as references.

Enzyme Assays

One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol of substrate per min.

The nitrilase activity of resting cells was determined in reaction mixtures (1.5–2 mL each) containing 50 mM sodium citrate buffer (pH 5.0), 10 mM mandelonitrile and an appropriate amount of cells. All nitrile stock solutions (200 mM–1 M) were prepared in methanol. The reaction mixtures were incubated at 30 °C. After different time intervals, samples (50–100 μL each) were taken and the reactions were stopped by the addition of 1 M HCl (5–10 μL). The samples were centrifuged in an Eppendorf centrifuge at 15,000 *g* for 10 min and the supernatants were analyzed using high-pressure liquid chromatography (HPLC).

Analytical Methods

Mandelonitrile, mandelic amide, and mandelic acid were analyzed by HPLC (HPLC Millenium Chromatography Manager 3.2, equipped with a diode array detector 996 and HPLC pump M510; Waters Associates, Milford, MA, USA). For the achiral analysis, a reversed-phase column (125 by 4 mm [internal diameter]; Trentec, Gerlingen, Germany) filled with 5-μm diameter particles of Lichrospher RP8 (E. Merck AG, Darmstadt, Germany) was used to identify individual compounds which were detected spectrophotometrically at 210 nm. The solvent system consisted of methanol (40% v/v) and H_3PO_4 (0.3% v/v) in H_2O. Separation of the enantiomers of mandelic acid and mandelic amide was achieved on a Chiral-HSA column (ChromTech AB, Hägersten, Sweden). The mobile phases consisted of sodium phosphate buffer (10 mM, pH 7.0) plus 4.5% (v/v) acetonitrile, respectively.

Acknowledgements

The presented work was supported by the DFG (partially in the framework of the CERC3 program) and the COST action CM0701.

References

[1] L. Martinková, V. Křen, *Biocatal. Biotransform.* **2002**, *20*, 73–93.
[2] H. Gröger, *Adv. Synth. Catal.* **2001**, *343*, 547–558.

[3] F. Effenberger, S. Förster, H. Wajant, *Curr. Opin. Biotechnol.* **2000**, *11*, 532–539.
[4] D. V. Johnson, A. A. Zabelinskaja-Mackova, H. Griengl, *Curr. Opin. Chem. Biol.* **2000**, *4*, 103–109.
[5] T. Ziegler, B. Hörsch, F. Effenberger, *Synthesis* **1990**, 575–578.
[6] S. Rustler, A. Müller, V. Windeisen, A. Chmura, B. Fernandes, C. Kiziak, A. Stolz, *Enzyme Microb. Technol.* **2007**, *40*, 598–606.
[7] A. Banerjee, P. Kaul, U. C. Banerjee, *Arch. Microbiol.* **2006**, *184*, 407–418.
[8] P. Kaul, A. Banerjee, S. Mayilraj, U. C. Banerjee, *Tetrahedron: Asymmetry* **2004**, *15*, 207–211.
[9] P. Kaul, A. Stolz, U. C. Banerjee, *Adv. Synth. Catal.* **2007**, *349*, 2167–2176.
[10] D. E. Robertson, J. A. Chaplin, G. DeSantis, M. Podar, M. Madden, E. Chi, T. Richardson, A. Milan, M. Miller, D. P. Weiner, K. Wong, J. McQuaid, B. Farwell, L. A. Preston, X. Tan, M. A. Snead, M. Keller, E. Mathur, P. L. Kretz, M. J. Burk, J. M. Short, *Appl. Environ. Microbiol.* **2004**, *70*, 2429–2436.
[11] a) K. Yamamoto, K. Oishi, I. Fujimatsu, K.-I. Komatsu, *Appl. Environ. Microbiol.* **1991**, *57*, 3028–3032; b) K. Yamamoto, I. Fujimatsu, K.-I. Komatsu, *J. Ferment. Bioeng.* **1992**, *73*, 425–430.
[12] M. Bauer, H. Griengl, W. Steiner, *Enzyme Microb. Technol.* **1999**, *24*, 514–522.
[13] A. Banerjee, R. Sharma, U. C. Banerjee, *Appl. Microbiol. Biotechnol.* **2002**, *60*, 33–44.
[14] U. Heinemann, D. Engels, S. Bürger, C. Kiziak, R. Mattes, A. Stolz, *Appl. Environ. Microbiol.* **2003**, *69*, 4359–4366.
[15] C. Kiziak, D. Conradt, A. Stolz, R. Mattes, J. Klein, *Microbiology* **2005**, *151*, 3639–3648.
[16] C. Mateo, A. Chmura, S. Rustler, F. van Rantwijk, A. Stolz, R. A. Sheldon, *Tetrahedron: Asymmetry* **2006**, *17*, 320–323.
[17] S. Rustler, H. Motejadded, J. Altenbuchner, A. Stolz, *Appl. Microbiol. Biotechnol.* **2008**, *80*, 87–97.
[18] H. Wajant, K. Pfizenmaier, *J. Biol. Chem.* **1996**, *271*, 25830–25834.
[19] B. Wilms, A. Wiese, C. Syldatk, R. Mattes, J. Altenbuchner, *J. Biotechnol.* **2001**, *86*, 19–30.
[20] H. Wajant, S. Förster, H. Böttinger, F. Effenberger, K. Pfizenmaier, *Plant Sci.* **1995**, *108*, 1–11.
[21] F. Effenberger, T. Ziegler, S. Förster, *Angew. Chem.* **1987**, *99*, 491–492.
[22] D. Costes, E. Wehtje, P. Adlercreutz, *Enzyme Microb. Technol.* **1999**, 25, 384–391.
[23] C. Kiziak, J. Klein, A. Stolz, *Protein Eng. Des. Sel.* **2007**, *20*, 385–396.
[24] R. Moser, *Diplomarbeit*, Universität Stuttgart, **1996**.
[25] T. Atlung, B. B. Christensen, F. G. Hansen, *Plasmid* **1999**, *41*, 110–119.
[26] a) M. Payne, S. Wu, R. Fallon, G. Tudor, B. Stieglitz, I. Turner, M. Nelson, *Biochemistry* **1997**, *36*, 5447–5454; b) R. Bauer, H.-J. Knackmuss, A. Stolz, *Appl. Microbiol. Biotechnol.* **1998**, *49*, 89–95; c) M. Hensel, S. Lutz-Wahl, L. Fischer, *Tetrahedron: Asymmetry* **2002**, *13*, 2629–2633.
[27] H. Bühler, F. Effenberger, S. Förster, J. Roos, H. Wajant, *ChemBioChem* **2003**, *4*, 211–216.
[28] J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular cloning: a laboratory manual*, 2nd edn., Cold Spring Harbor Press, Cold Spring Harbor, N.Y., **1989**.
[29] C. T. Chung, S. L. Niemela, R. H. Miller, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 2172–2175.
[30] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248–254.
[31] U. K. Laemmli, *Nature* **1970**, *227*, 680–685.

