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Characterisation of the substrate specificity of the nitrile hydrolyzing system of the acidotolerant black yeast *Exophiala oligosperma* R1

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Abstract: The 'black yeast' *Exophiala oligosperma* R1 can utilise various organic nitriles under acidic conditions as nitrogen sources. The induction of a phenylacetonitrile converting activity was optimised by growing the strain in the presence of different nitriles and /or complex or inorganic nitrogen sources. The highest nitrile hydrolysing activity was observed with cells grown with 2-cyanopyridine and $NaNO_3$. The cells metabolised the inducer and grew with 2-cyanopyridine as sole source of nitrogen. Cell extracts converted various (substituted) benzonitriles and phenylacetonitriles. They usually converted the isomers carrying a substituent in the *meta*-position with higher relative activities than the corresponding *para*- or *ortho*-substituted isomers. Aliphatic substrates such as acrylonitrile and 2-hydroxy-3-butenenitrile were also hydrolysed. The highest specific activity was detected with 4-cyanopyridine. Most nitriles were almost exclusively converted to the corresponding acids and no or only low amounts of the corresponding amides were formed. The cells hydrolysed amides only with extremely low activities. It was therefore concluded that the cells harboured a nitrilase activity. The specific activities of whole cells and cell extracts were compared for different nitriles and evidence obtained for limitation in the substrate-uptake by whole cells. The conversion of 2-hydroxy-3-butenenitrile to 2-hydroxy-3-butenoic acid at pH 4 demonstrated the unique ability of cells of *E. oligosperma* R1 to hydrolyse aliphatic α-hydroxynitriles under acidic conditions. The organism could grow with phenylacetonitrile as sole source of carbon, energy and nitrogen. The degradation of phenylacetonitrile presumably proceeds via phenylacetic acid, 2-hydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid (homogentisate), maleylacetoacetate and fumarylacetoacetate.

Key words: Acidotolerance, biotransformation, black yeasts, *Exophiala*, homogentisate pathway, induction, nitrilase.

INTRODUCTION

Organic nitriles (R-CN) are widely used in organic chemistry as intermediates for the synthesis of various compounds such as carboxylic acids and amides (Banerjee *et al*. 2002). Since several yr there is an increasing interest in identifying and using nitrile converting biocatalysts in order to replace the traditional chemical synthetic reactions for the production of acids and amides which require rather harsh acidic or alkaline conditions (Singh *et al*. 2006). Furthermore, several examples have been described that demonstrate that nitrile-converting biocatalysts allow chemo-, regio-, or enantioselective reactions that are difficult to achieve by purely chemical reactions (Bunch 1998a, Martínková & Křen 2001, Schulze 2002, Brady *et al*. 2004). These investigations resulted in the isolation of several nitriles converting Gram-positive and Gram-negative bacteria (often *Rhodococcus* or *Pseudomonas* strains) and some yeasts and fungi (Banerjee *et al*. 2002, Bunch 1998b, Kaplan *et al*. 2006a, b, Kaul *et al.* 2004, Kiziak *et al*. 2005, Rezende *et al*. 1999). These organisms converted nitriles either to the acids by using nitrilases or to the amides by using nitrile hydratases.

An interesting application for nitrile hydrolysing organisms or enzymes is the conversion of α-hydroxynitriles, because (chiral) α-hydroxycarboxylic acids and amides are interesting products for the chemical industry (Gröger 2001). Unfortunately, the biotransformation of α-hydroxynitriles in aqueous systems is hampered by the low stability of the substrates under neutral conditions. In contrast, these substrates are generally more stable under acidic conditions (Rustler *et al*. 2007). However, most of the known nitrilases are unstable at acidic pH values (Banerjee *et al.* 2002). Therefore, we are trying to find whole cell catalysts which are able to catalyse these biotransformations under acidic conditions. We have recently isolated after an enrichment at pH 4 with phenylacetonitrile as sole source of nitrogen an acidotolerant black yeast which was subsequently identified as a new strain of *Exophiala oligosperma* (Rustler & Stolz 2007). This strain (R1) was the first 'black yeast' for which the ability to convert organic nitriles was described and it was found that the organism could grow at pH 4 with phenylacetonitrile as sole source of carbon, nitrogen and energy. Although this nitrile converting system showed some biotechnological potential because of the unique taxonomic position of the producing organism and the acid-resistance of the process, the observed activities were not sufficient for a detailed analysis of the reaction and any possible application. Therefore, in the present communication we further optimised the induction conditions for the production of the nitrile converting activity and subsequently analysed the responsible enzymatic activity.

MATERIALS AND METHODS

Microorganisms and culture conditions

Exophiala oligosperma R1 [deposited at the Centraalbureau voor Schimmelcultures in Utrecht (The Netherlands) as strain CBS 120260] was routinely grown in Na-citrate-phosphate mineral media consisting of 20 % (*v/v*) 0.5 M Na-citrate-phosphate buffer (pH 4) and the nitrogen-free mineral medium described previously (Rustler & Stolz 2007) plus 0.2 % (*w/v*) casamino acids and 20 mM

glucose as sources of nitrogen, carbon and energy. The cultures were usually grown for 72–120 h in 3 L Erlenmeyer flasks with baffles at 30 °C on a rotary shaker (100 rpm).

Growth measurements

The growth of the strain was monitored spectrophotometrically by measuring the optical density at 600 nm (OD_{600nm}) with a Cary 100 Bio spectrophotometer (Varian Inc., Mulgrave, Australia) or by using a Klett photometer (Klett Manufactoring Co. Inc., Brooklyn, NY). An OD_{600nm} of 1 corresponded to about 75 Klett units and 0.25 mg of cell dry weight per mL of culture.

Preparation of cell extracts

The cells were harvested at the beginning of the stationary growth phase by centrifugation (11,000 g, 15 min, 4 °C) and washed twice in 0.1 M Na-phosphate buffer (pH 6.5). The cells were then resuspended in 0.1 M Tris/HCl buffer (pH 7) to an OD_{600nm} of about 300 and disintegrated by using a French Press (Aminco, Silver Springs, Md., U.S.A.). The cells were disintegrated seven times at 4 °C by using the miniature pressure cell (Aminco, Silver Springs, Md., U.S.A.) with a cell pressure of about 16 000 psi. The samples were centrifuged (6 000 g, 10 min, 4 °C) and the liquid phase was finally clarified by ultracentrifugation (100 000 g, 60 min, 4 °C). The supernatant was immediately frozen in liquid nitrogen and stored at –70 °C. The protein content of the cell extract was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Enzyme assays

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

For the determination of nitrile hydrolysis with resting cells, the cells were harvested at the beginning of the stationary phase by centrifugation (11 000 g, 15 min, 4 °C), washed twice in 0.1 M Na-citrate-phosphate buffer (pH 4) and finally resuspended in 0.1 M Na-citrate-phosphate buffer (pH 4). The standard assay usually contained 1.5–2 mL 0.1 M Na-citrate-phosphate buffer (pH 4), 1–10 mM substrate and cells corresponding to an OD_{600nm} of 2–15. The reactions were started by the addition of the substrates and performed in Eppendorf cups incubated in a thermoshaker at 30 °C and 1 400 rpm (Thermomixer Comfort, Eppendorf AG, Hamburg, Germany). The stock solutions (1 M) of the nitriles (or amides) were routinely prepared in methanol (or a 1:1 mixture of methanol and water for some amides). After different time intervals, samples (50–100 µL each) were taken and the reactions were stopped by adding 1 M HCl (5–10 µL). The cells were removed by centrifugation (21 000 *g*, 10 min, 4 °C) and the supernatants finally analysed by using high pressure liquid chromatography (HPLC).

The nitrile hydrolysing activity of cell extracts was determined in reaction mixtures (0.75–1 mL) containing 0.1 M Tris/HCl (pH 7), 0.15–0.2 mg/mL of protein and 1–5 mM of the substrate. The reactions were then performed as described for the resting cell experiments. The reactions were usually terminated by the addition of 10 % (*v/v*) 1 M HCl and the precipitated proteins were removed by centrifugation (21 000 g, 10 min, 4 °C). The concentrations of the substrates and products in the supernatants were determined by HPLC.

The activities were usually calculated from the turn-over of the substrate and correlated to the dry weight of the cells or the amount of protein applied.

Comparison of the conversion of different nitriles by resting cells and cell extracts

Exophiala oligosperma R1 was cultivated in 3 L Erlenmeyer flasks with baffles in 1 L of the Na-citrate-phosphate mineral medium (pH 4) containing glucose (20 mM), $NaNO_3$ (4 mM), and 2-cyanopyridine (15 mM). The cultures were incubated at 30 °C on a rotary shaker (100 rpm). At the end of the exponential growth phase (after 72 h of cultivation; $OD_{600nm} \approx 3.5$) the cells were harvested by centrifugation (11 000 g, 15 min, 4 °C) and washed in 0.1 M Na-phosphate buffer (pH 6). An aliquot of the cells was immediately frozen in liquid nitrogen and stored at –70 °C. The other aliquot was resuspended in 0.1 M Tris/HCl (pH 7) and used for the preparation of cell extracts as described above. For the experiments cells and cell extracts were thawed and resuspended or diluted, respectively, in 0.1 M Tris/HCl (pH 7). The reaction mixtures finally contained in 0.1 M Tris/HCl (pH 7) resting cells with an OD_{600nm} of 2 or a protein concentration of 0.2 mg/mL in case of the cell extracts.

Analytical methods

The concentrations of the nitriles and their corresponding amides and acids were analysed by HPLC (ChemStation LC3D, Autosampler G1329A, Thermostat 1330B, Diode Array Detector G1315B, Quat-HPLC pump G1311A; Agilent Technologies, Santa Barbara, CA). The individual compounds were usually detected spectrophotometrically at 210 nm.

A reversed-phase column [125 by 4 mm (internal diam); Trentec, Gerlingen, Germany] filled with 5-µm-diameter particles of Lichrospher-RP8 endcapped (E. Merck AG, Darmstadt, Germany) was used in most experiments for separation of individual compounds. The conversion of cyanopyridines, acrylonitrile and 2-hydroxy-3-butenenitrile was analysed by using a column [size 250 by 4 mm (internal diameter) Trentec, Gerlingen, Germany] filled with 5 µm particles of Nucleosil-100 C18 (Macherey & Nagel, Düren, Germany). The columns were incubated at 21 °C in a column heater /chiller (Jones Chromatography Model 7956, Alltech Associates Inc., Hesperia, CA) and the samples were cooled at 4 °C.

The solvent systems for the analysis of the turn-over experiments with benzonitrile, 2-, 3-, and 4-tolunitrile, 2-, 3-, and 4-chlorobenzonitrile, 2-, 3-, and 4-hydroxybenzonitrile, phenylacetonitrile, 2-phenylpropionitrile and 2-, 3-, and 4-chlorophenylacetonitrile contained 30–50 % (v/v) acetonitrile and 0.3 % H_3PO_4 (v/v) in H_2O as mobile phases. The conversion of mandelonitrile was analysed by using a solvent system which consisted of 40 % (v/v) methanol, 0.3 % (v/v) H_3PO_4 and water.

Acrylonitrile, 2-hydroxy-3-butenenitrile and their potential products were analysed by using a solvent system consisting of 0.5 % (v/v) acetonitrile, 0.1 % (v/v) H_3PO_4 and 99.4 % (v/v) H_2O. The detection of these aliphatic substances was performed at 195 nm.

2-, 3-, and 4-cyanopyridine were analysed by using a solvent system which contained 20 % (v/v) acetonitrile and 0.3 % (v/v) H_3PO_4 in water by using a Lichrospher-RP8 column. The products formed from 3- and 4-cyanopyridine were detected by using a solvent system consisting of 10 % (v/v) acetonitrile plus 90 % (v/v) 30 mM Na-PO_4-buffer (pH 7) and 5 mM of the ion-pair reagent TBAHS (tetrabutylammoniumhydrogensulfate) and by using a

Nucleosil-100 C18 column as the stationary phase (Dazzi *et al*. 2001).

Products formed from 2-cyanopyridine were analysed with a Nucleosil-100 C18 column using a solvent system composed of 99 % (v/v) 30 mM Tris/HCl (pH 9.0) plus 1 % (v/v) acetonitrile and 5 mM TBAHS. The detection of substances which were separated in solvent systems with TBAHS was performed at 265 nm. The average flow rate was 1 mL/min.

Synthesis of 2-hydroxy-3-butenenitrile

Into a 250 mL round bottom flask 0.09 mol (5 g) acrolein, 0.1 mol (9.9 g) trimethylsilyl cyanide and 0.01 mol (3.19 g) zinc iodide were added, followed by addition of 200 mL dichloromethane. The reaction was stirred overnight at RT. Then, the solvent was evaporated under reduced pressure, giving the silyl protected cyanohydrin. The latter was hydrolyzed at 40 °C to 2-hydroxy-3-butenenitrile by reacting it with 150 mL 3 M HCl. The reaction was completed after 2 h. The pure product (dark brown liquid) was isolated from the reaction mixture by triple extraction with diethyl ether (3 x 150 mL), drying on $MgSO_4$ and concentrating the product using a rotary evaporator (Gassman and Talley 1978). Proton NMR analysis confirmed the product formation. ^{1}H NMR (300 MHz, $CDCl_3$): δ= 5.9 (ddd, 2H, *J'*=16.2 *J'*=11.1, *J'*=6.3 Hz), 5.6 (d, 3 H, *J*=17.1), 5.4 (d, 3 H,*J* =10.2), 5.0 (d, 1 H, *J* =5.1).

Synthesis of 2-hydroxy-3-butenoic amide

Into a 10 mL glass reactor, 12 mmol (1 g) 2-hydroxy-3-butenenitrile and 3.2 mL 37 % HCl were added. The reaction was shaken for 3.5 h at 10 °C until the cyanohydrin was completely converted into the corresponding 2-hydroxy-3-butenoic amide. The latter product was extracted to ethyl acetate, dried on Na_2SO_4 and the resulting clear solution was concentrated using a rotary evaporator (van Langen *et al*. 2004). The formation of the product was confirmed by proton NMR. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.9 (bs, $CONH_2$), 6.2 (ddd, 2H, *J'*=16.8, *J'*=11.4, *J'*=6.6 Hz), 5.5 (d, 3 H, *J*=16.5), 5.3 (d, 3 H, *J*=10.5), 4.7 (d, 1H, *J*=5.7 Hz).

Synthesis of 2-hydroxy-3-butenoic acid

Into a 10 mL round bottom flask, 6 mmol (0.5 g) 2-hydroxy-3-butenoic amide and 3.0 mL 37 % HCl were added. The reaction was refluxed at 125 °C for 1.5 h. 2-hydroxy-3-butenoic acid was extracted to ethyl acetate, dried on Na_2SO_4 and the product was concentrated *in vacuo* (van Langen *et al*. 2004). The formation of the product was confirmed by proton NMR. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.0 (ddd, 2H, *J'*=15.4, *J'*=11.4, *J'*=6.6 Hz), 5.5 (d, 3H, *J*=17.4), 5.3 (d, 3H,*J*=10.5,), 4.8 (m,1H).

Chemicals

Trimethylsilyl cyanide and zinc iodide (98+ %) were obtained from Acros Organics BVBA (Geel, Belgium). All other chemicals were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) or E. Merck AG (Darmstadt, Germany). All the chemicals were used as supplied, without further purification.

RESULTS

Induction of the nitriles converting activity after addition of different possible inducers

There are several examples found in the literature which demonstrate that the addition of certain nitriles may have pronounced inducing effects on nitriles converting enzymes. Thus, the addition of ε-caprolactam or isovaleronitrile resulted in *Rhodococcus rhodochrous* J1 and *R. rhodochrous* K22 in the formation of relatively large amounts of the respective nitrilases which corresponded to 20–30 % of the total soluble protein (Nagasawa *et al*. 1990, Kobayashi *et al.* 1991). Recently, 2-cyanopyridine and valeronitrile have been described as especially potent inducers of the nitrilase from *Aspergillus niger* K10 (Kaplan *et al.* 2006b). Therefore, the influence of these and structural similar compounds was assayed on the induction of the nitrile converting activity of *E. oligosperma* R1. The strain was grown in Na-citrate-phosphate medium (pH 4) with glucose (20 mM), casamino acids (0.2 % w/v) and different potential inducers: phenylacetonitrile, 2-, 3-, and 4-chlorophenylacetonitrile, indole-3-acetonitrile, 4-hydroxyphenylacetonitrile, 2-, 3-, and 4-cyanopyridine, isovaleronitrile, or benzonitrile (2 mM each). Furthermore, also ε-caprolactam (45 mM) was analysed.

Exophiala oligosperma R1 did not grow in the presence of the indicated concentrations of 2-, 3-, and 4-chlorophenylacetonitrile and indole-3-acetonitrile. In contrast, no inhibitory effects of the other compounds were observed on growth. The cells were harvested at the beginning of the stationary growth phase by centrifugation (11,000 *g*, 15 min, 4 °C), washed, and resupended in 0.1 M Na-citrate-phosphate buffer (pH 4) to an OD_{600nm} of 11. Finally, phenylacetonitrile (2 mM) was added and the conversion of the nitrile analysed by HPLC.

The experiments demonstrated that the addition of 2-cyanopyridine, ε-caprolactam, and isovaleronitrile increased the nitrile converting activity compared to the constitutive level 4.4-, 2.9-, and 2-fold, respectively. In contrast, the previously used inducer phenylacetonitrile resulted only in a 1.5-fold increase in enzyme activity. Also the other tested putative inducers only resulted in a less than 1.5-fold increase in the nitrile converting activity.

Influence of different nitrogen sources on induction and optimisation of the inducer concentrations

In the following experiment the influence of different nitrogen sources on the induction of the nitriles converting activity was assayed. The cells were grown in Na-citrate-phosphate medium (pH 4) with glucose (20 mM) plus casamino acids (0.2 % *w/v*), $NaNO_3$ (3 mM) or $(NH_4)_2SO_4$ (1.5 mM) with or without phenylacetonitrile (2 mM), 2-cyanopyridine (2 mM), ε-caprolactam (45 mM), or a mixture of 2-cyanopyridine plus isovaleronitrile (2 mM each). In these experiments the highest activities were obtained in a growth medium containing $(NH_4)_2SO_4$ plus ε-caprolactam (45 mM), followed by a growth medium which contained $NaNO_3$ plus 2-cyanopyridine.

In the previous experiments fixed concentrations of the inducers (2 mM of the nitriles or 45 mM ε-caprolactam) were used. In the following experiments the concentrations of the previously identified best inducers (ε-caprolactam and 2-cyanopyridine) were varied (2–45 mM). The cells were grown in 300 mL Klett-flasks with baffles in 50 mL of a medium which contained Na-citrate-phosphate buffer (pH 4), nitrogen-free mineral medium, 20 mM glucose. Furthermore,

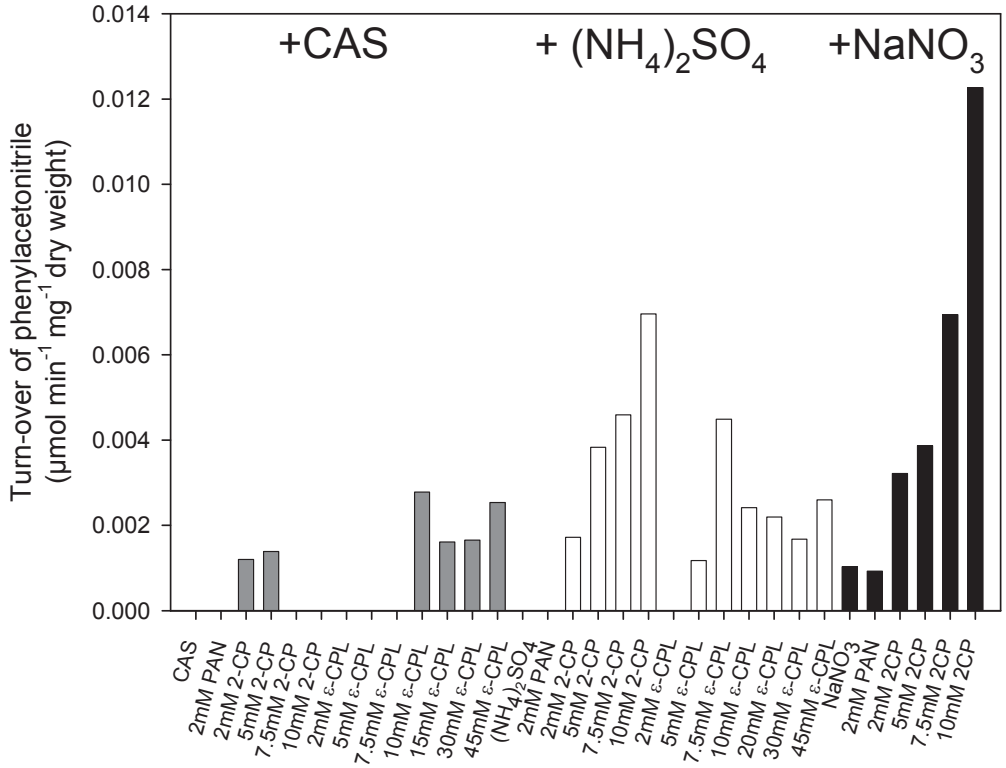

Fig. 1. Nitrile hydrolyzing activity of resting cells of *E. oligosperma* R1 after growth in the presence of different nitrogen sources and different concentrations of ε-caprolactam or 2-cyanopyridine. The cells were cultivated as described in the text in media containing Na-citrate buffer (pH 4), glucose (20 mM) and 0.2 % (*w/v*) casamino acids (CAS; ■), 4 mM $NaNO_3$ (■), or 2 mM $(NH_4)_2SO_4$ (□). The cells were harvested by centrifugation (11 000 g, 15 min, 4 °C), washed in 0.1 M Na-citrate-phosphate buffer (pH 4), and finally resuspended in 0.1 M Na-citrate-phosphate buffer (pH 4) to an OD_{600nm} of approximately 15. The conversion of phenylacetonitrile (2 mM) by the resting cells was analysed using a thermoshaker (30 °C, 1 400 rpm) as described in the materials and methods section.

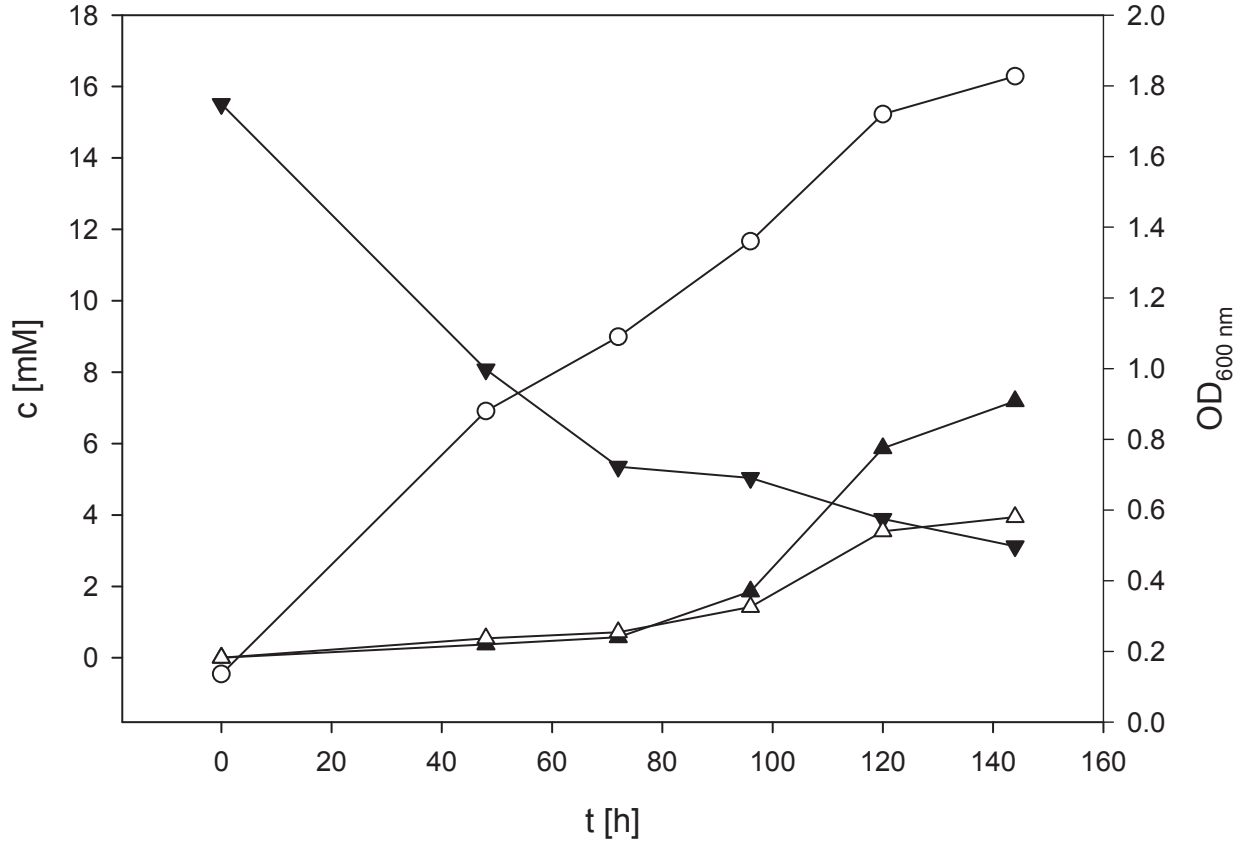

Fig. 2. Turn-over of 2-cyanopyridine by growing cells of *E. oligosperma* R1**.** The cells were grown in Na-citrate-phosphate medium (pH 4) with glucose (20 mM), $NaNO_3$ (4 mM), and 2-cyanopyridine (15 mM). The cells were incubated in Erlenmeyer flasks on a rotary shaker (100 rpm) at 30 °C. The growth of the culture was monitored by measuring the optical density at 600 nm (O). After different time intervals samples were taken and the reactions terminated by adding 10 % (*v/v*) 1 M HCl. Cells were removed by centrifugation (21 000 g, 10 min, 4 °C) and the concentration of 2-cyanopyridine (▼), picolinic acid (▲), and picolinic amide (Δ) in the supernatants determined by HPLC.

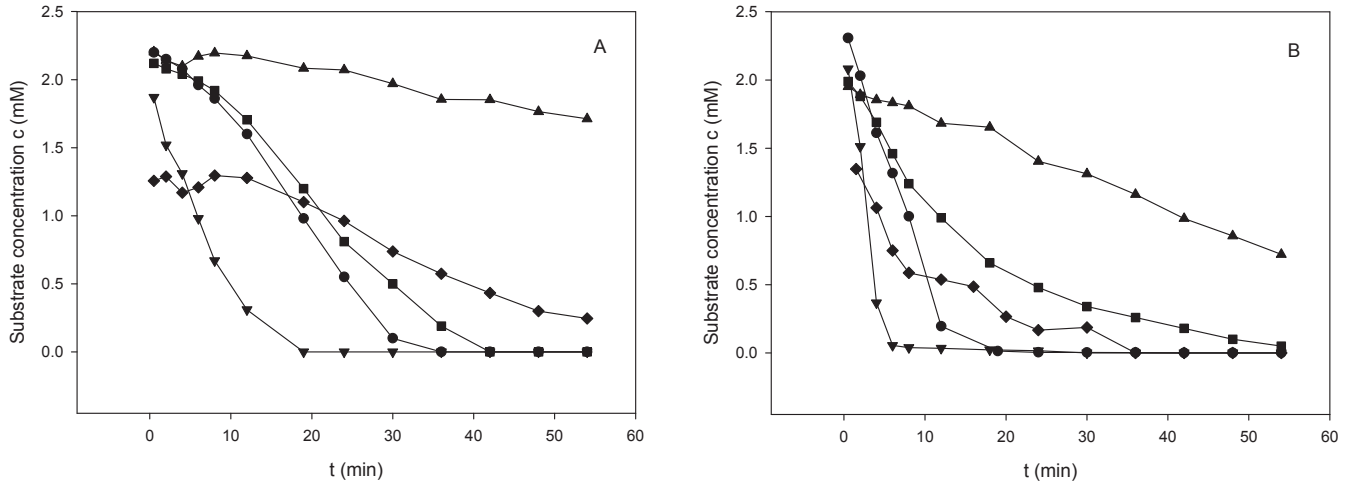

Fig. 3. Turn-over of different nitriles by whole cells (A) and cell extracts (B) of *E. oligosperma* R1**.** The resting cells and cell extracts were prepared as described in the materials and methods section. The reaction mixtures (750 µL each) were incubated in 1.5 mL cups in a thermoshaker (1 400 rpm, 30 °C). The reactions were started by the addition of 4-cyanopyridine (▼), 3-cyanoypridine (●), benzonitrile (■), phenylacetonitrile (▲) (2 mM each), or acrylonitrile (♦; 1.5 mM). After different time intervals samples were taken and the reactions terminated by adding 20 % (*v/v*) 1 M HCl. The samples were clarified by centrifugation (21 000 g, 10 min, 4 °C), and the supernatants analysed by HPLC (see material and methods).

casamino acids (0.2 % *w/v*), $(NH_4)_2SO_4$ (2 mM), or $NaNO_3$ (4 mM) were offered as nitrogen sources. The cells were harvested at the end of the exponential growth phase (after 96 h with casamino acids or after 112 h with inorganic nitrogen sources). The cells reached in the presence of the casamino acids significantly higher final cell densities than after growth with the inorganic nitrogen sources (OD_{600nm} of about 6.5 or 3.5, respectively). In both systems (inorganic or organic nitrogen sources) the addition of ε-caprolactam and 2-cyanopyridine did not result in significant decreases in the finally reached optically densities. The results demonstrated that with ε-caprolactam the highest nitrile hydrolysing activities were found with 7.5 mM ε-caprolactam and $(NH_4)_2SO_4$. In these experiments 2-cyanopyridine was a better inducer than ε-caprolactam. Almost twice as much activity could be detected in samples that had been grown with $NaNO_3$ and 10 mM 2-cyanopyridine (Fig. 1).

Conversion of 2-cyanopyridine by the cells

The analysis of further induction experiments with 2-cyanopyridine demonstrated that the cells metabolised the inducer. Thus, in the presence of 4 mM $NaNO_3$ an (almost) complete disappearance of 2-CP (10–15 mM) was observed within 96–144 h. The analysis of these reactions by HPLC [solvent system: 1 % (*v/v*) acetonitrile and 99 % (*v/v*) 30 mM Tris/HCl, pH 9.0, plus 5 mM TBAHS, see materials and methods] demonstrated the turn-over of 2-CP (R_t= 19.4 min) and the formation of two metabolites. These metabolites were according to their retention times and *in situ* spectra identified as picolinic acid (pyridine-2-carboxylic acid) (R_t= 11.2 min) and picolinic amide (2-pyridinecarboxamide) (R_t= 10.5 min) (Fig. 2). The strain also grew in the absence of $NaNO_3$ with 10–15 mM 2-CP as sole source of nitrogen and also under these conditions almost completely converted the nitrile with similar specific activities. Further, resting cell experiments with cells grown with 2-cyanopyridine (12.5 mM) in the absence or presence of $NaNO_3$ (4 mM) did not show any significant differences in the ability to convert phenylacetonitrile.

Identification of better substrates for the nitrile converting enzyme

The optimisation of the induction conditions described above resulted in an approximately 20-fold increase of the nitrile-converting activity compared to the initially found values (for cells grown with phenylacetonitrile and casamino acids) (Rustler & Stolz 2007). Nevertheless, even these 'optimised' cells (grown in Na-citrate-phosphate medium with 4 mM $NaNO_3$ and 15 mM 2-cyanopyridine) demonstrated with phenylacetonitrile as substrate only activities of about 0.021 U per mg of dry weight. It was therefore tested if nitriles which had been previously identified as good substrates for fungal nitrilases (Kaplan *et al*. 2006a, Goldlust & Bohak 1989) were converted with higher specific activities. Therefore, whole resting cells were incubated with benzonitrile, 3-, and 4-cyanopyridine (2 mM each) and acrylonitrile (1.5 mM) (Fig. 3A).

The experiments demonstrated that the resting cells converted 3- and 4-cyanopyridine about 7 and 13-times faster, respectively, than phenylacetonitrile (Table 1). The resting cells converted 4-cyanopyridine after induction with 2-cyanopyridine with specific activities of 0.27 U/mg of dry weight. This represented an approximately 270-fold increase compared to the specific activity previously reported for the conversion of phenylacetonitrile by cells grown with casamino acids plus phenylacetonitrile (Rustler & Stolz 2007). The experiments further showed that benzonitrile and acrylonitrile were also converted with higher activities than phenylacetonitrile.

Comparison of the nitrile hydrolysing activity of whole cells and cell extracts

In the following experiments it was tested if the uptake of the substrates could be a limiting factor for the conversion of nitriles by *E. oligosperma* R1. Therefore, cell extracts were prepared and the conversion of benzonitrile, phenylacetonitrile, 3-, and 4-cyanopyridine and acrylonitrile compared between resting cells and cell extracts (Fig. 3). The experiments demonstrated that the cell extracts exhibited significant activities for the transformation of the cyanopyridines and specific activities of cell extracts for

Table 1. Conversion of different nitriles by whole cells and cell extracts of *E. oligosperma* R1.

	Activity of whole cells		Activity of cell extracts	
Compound	**U/mg of dry weight**	**Relative activity**	**U/mg of total protein**	**Relative Activity**
Benzonitrile	0.115	100	0.48	100
Phenylacetonitrile	0.021	18	0.11	22
3-Cyanopyridine	0.14	124	1	208
4-Cyanopyridine	0.27	232	2.46	512
Acrylonitrile	0.058	50	0.58	121

The specific activities were calculated from the experiments shown in Fig. 3 based on the decrease of the nitrile concentrations and related to the dry weight of the cells or the protein concentration in the experiments with cell extracts, respectively.

the conversion of 4-cyanopyridine up to 2.5 U/mg of protein were calculated (Table 1). In the literature generally protein contents of 39–56 % (expressed as % of dry weight) have been described for different yeast species (Verduyn 1991). An average of 50 % protein content (related to the dry weight) was used for the comparison of the turn-over-rates of cell extracts and resting cells. Thus, it was calculated that the cell extracts converted 3-, and 4-cyanopyridine as well as acrylonitrile with up to 5-times higher specific activities than the resting cells. This suggested that at least in the case of rapidly converted substrates the uptake of the nitriles has a limiting effect on the *in vivo* metabolism of organic nitriles by the yeast cells. Furthermore, also the comparison of the relative activities of the whole cells and the cell extracts with different nitriles suggested a significant influence of the cell membrane on the nitrile metabolism because the whole cells converted the cyanopyridines and acrylonitrile in comparison to benzonitrile with decreased relative activities (Table 1). This was especially evident for the substrates benzonitrile and acrylonitrile, because crude extracts converted acrylonitrile faster than benzonitrile, while this was the opposite in the whole cell system.

Conversion of different nitriles by cell extracts

The substrate specificity of the nitrile hydrolysing activity was determined with cell extracts and compared to other fungal nitrile converting enzymes (Table 2). These experiments demonstrated that cell extracts from *E. oligosperma* R1 converted in addition to benzonitrile and the cyanopyridines various methyl-, chloro-, and hydroxy-substituted benzonitriles and phenylacetonitriles. The cell extracts converted in general all isomers of a given substituted benzonitrile or phenylacetonitrile. Nevertheless, it became evident that in most cases the *meta*-substituted isomers were converted with the highest relative activities. In most cases the *meta*-substituted benzonitriles were even faster converted than benzonitrile. In contrast, the *ortho*-substituted isomers were generally converted with the lowest relative activities.

Phenylacetonitriles which carried a larger substituent at the α-position than a hydrogen atom (such as mandelonitrile and 2-phenylpropionitrile) were converted with significantly reduced conversion rates. The aliphatic substrate acrylonitrile was converted with high relative activities (121 % compared to benzonitrile).

The conversion of the nitriles resulted in almost all experiments (with the exceptions of 2-chlorobenzonitrile, 4-cyanopyridine, and acrylonitrile as substrates) in the formation of one clearly prominent product which represented (according to its signal intensity during the HPLC analysis) more than 95 % of the products formed (Table 2). These products were identified by the comparison with authentic standards by their retention times and *in situ* UV/VIS spectra as the corresponding acids. In the cases of 4-cyanopyridine and acrylonitrile two products were formed in a ratio of about 9:1. The conversion of 2-chlorobenzonitrile resulted in two signals in a ratio of 7:3. However, also for these substrates it was shown by using authentic standards that the prominent products were the corresponding acids. The minor products were identified according to their retention times as the corresponding amides.

The conversion of 2-cyanopyridine and 2-hydroxybenzonitrile did not result in the formation of detectable amounts of products. However, in the case of 2-cyanopyridine the conversion of the substrate to picolinic acid and picolinic amide had already been observed in the growth experiment described before (see Fig. 2). The cell extracts were also incubated with several amides, which would be intermediately formed if the cells would harbour a nitrile hydratase. The experiments showed that picolinic amide and nicotinic amide were not converted and isonicotinic amide only with extremely low activities to the corresponding acid. This indicated only a rudimental amidase activity and gave strong evidence that the nitriles were indeed converted by a nitrilase activity.

Conversion of 2-hydroxy-3-butenenitrile by whole cells of *E. oligosperma* R1

It was previously shown that resting cells of *E. oligosperma* R1 were able to convert nitriles at pH values ≥1.5 (Rustler & Stolz 2007). This could allow the conversion of α-hydroxynitriles, which are unstable at neutral pH-values but stabilised under acidic conditions by resting cells of *E. oligosperma* R1. The conversion of acrylonitrile by whole cells and cell extracts demonstrated the general ability of *E. oligosperma* R1 to convert aliphatic nitriles with high relative reaction rates. Therefore, 2-hydroxy-3-butenenitrile was synthesised as substrate (see material and method section) and incubated in a reaction mixture containing 0.1 M Na-citrate-phosphate buffer (pH 4) and resting cells of *E. oligosperma* R1. The analysis of the reaction mixture demonstrated a disappearance of almost 50 % of the initial amount of 2-hydroxy-3-butenenitrile within the first 80 min of the reaction and the formation of one product (Fig. 4). In contrast, no significant decrease of 2-hydroxy-3-butenenitrile was observed in a control experiment without cells. Only traces of acroleine (2-propenal, acrylaldehyde) which would be formed by the chemical decomposition of 2-hydroxy-3-butenenitrile were detected in the reaction mixtures with and without cells. Thus, it was concluded that 2-hydroxy-3-butenenitrile was almost completely stabilised by the acidic reaction buffer and that the resting cells indeed converted the nitrile. The product formed from 2-hydroxy-3-butenenitrile by the cells was identified according to its retention time (R_t=3.45 min) and UV/VIS spectrum in comparison with a chemically synthesised authentic standard as 2-hydroxy-3-butenoic acid.

Table 2. Comparison of the relative nitrile hydrolysing activities of cell extracts from *Exophiala oligosperma* R1, *Fusarium solani* O1, *Penicillium multicolor* CCF 2244 and the purified nitrilase from *Aspergillus niger* K1.

Substrate	*E. oligosperma* R1		*P. multicolor* CCF 2244[1] (whole cells)	*F. solani* O1[2] (purified enzyme)	*A. niger* K1[3] (purified enzyme)
	Relative activity (%)	Formation of amide (% of total products)	Relative activity (%)	Relative activity (%)	Relative activity (%)
Benzonitrile	100	nd	100	100	100
2-Tolunitrile	≤5	nd	nd	nd_m	nd
3-Tolunitrile	154	nd	11.3	33	5.5
4-Tolunitrile	16	nd	nd	16	3.4
2-Hydroxybenzonitrile	≤5	nd	nd	nd_m	nd
3-Hydroxybenzonitrile	140	nd	0.8	80	5.8
4-Hydroxybenzonitrile	10	nd	nd	3	nd
2-Chlorobenzonitrile	<10	29	nd	nd_m	nd
3-Chlorobenzonitrile	100	<5	14.3	87	41
4-Chlorobenzonitrile	49	nd	2.8	40	29.8
2-Cyanopyridine	<10	nd	40	nd_m	14.2
Picolinic amide	nd	-	nd	nd_m	-
3-Cyanopyridine	208	nd	15	28	32.4
Nicotinic amide	nd	-	18	nd_m	-
4-Cyanopyridine	566	<10	72	130	410.7
Isonicotinic amide	<5	-	nd	nd_m	-
Phenylacetonitrile	19	nd	2.3	nd_m	10.8
2-Chlorophenylacetonitrile	11	nd	nd_m	nd_m	nd_m
3-Chlorophenylacetonitrile	35	nd	nd_m	nd_m	nd_m
4-Chlorophenylacetonitrile	22	nd	nd_m	nd_m	nd_m
Mandelonitrile	<5	Traces	nd_m	nd_m	nd_m
2-Phenylpropionitrile	<10	nd	nd	nd	1
Acrylonitrile	121	<10	nd_m	nd_m	nd_m

Cell extracts of *E. oligosperma* R1 were produced as described in the materials and methods section. The reaction mixtures (750 µL each) contained 0.1 M Tris/HCl (pH 7) and 0.2 mg/mL of protein and were incubated in 1.5 mL cups in a thermoshaker (30 °C, 1 400 rpm). The reactions were started by the addition of the respective substrates (2 mM each). At different time intervals samples were taken, and the reactions terminated by the addition of 20 % (*v/v*) 1 M HCl. The reaction mixtures were centrifuged (21 000 g, 10 min, 4 °C) and the supernatants analysed by HPLC according to the procedures described in the material and method section. The reaction rates were calculated from the turn-over of the substrates. The activitiy of benzonitrile was taken as 100 % (0.48 U/mg of protein).
[1,2,] The data for the *Penicillium,* and the *Aspergillus* strains were adapted from Kaplan *et al.* (2006a, c) and [3] those for the *Fusarium* strain from Vejvoda *et al.* 2008, nd: not detected; nd_m: not determined

Degradation of aromatic compounds by *E. oligosperma* R1

It was previously demonstrated that *E. oligosperma* R1 could grow with phenylacetonitrile as sole source of carbon, energy, and nitrogen and that resting cells converted phenylacetonitrile via phenylacetate to 2-hydroxyphenylacetate, which finally also disappeared from the culture supernatants. This suggested that *E. oligosperma* R1 could further metabolise 2-hydroxyphenylacetate and finally cleave the aromatic ring (Rustler & Stolz 2007). Therefore, *E. oligosperma* R1 was grown with phenylacetonitrile as sole source of carbon and energy, the cells harvested by centrifugation and resting cells ($OD_{546nm} \cong 200$) incubated with 2 mM 2-hydroxyphenylacetate and different inhibitors of Fe(II)-ions containing ring-fission dioxygenases (ortho-phenanthroline, 8-hydroxyquinoline, or 2,2´-bipyridyl, 1 mM each). The reactions were analysed by HPLC (40 % methanol, 0.3 % H_3PO_4 in water, flow rate 0.6 mL/min) and it was found that the cells converted 2-hydroxyphenylacetate (R_t= 5.0 min) in the presence of all three inhibitors to a product which was identified in comparison with an authentic standard according to its retention time (R_t= 2.8 min) and *in situ* spectrum (λ_{max}= 222 nm, 292 nm) as 2,5-dihydroxyphenylacetate (homogentisate). In order to analyse the further metabolism of homogentisate, cell extracts were prepared from cells of *E. oligosperma* R1 grown with phenylacetonitrile as sole source of carbon and energy. The cell extracts were incubated in 25 mM Tris/HCl (pH 7.4) with 0.1 mM homogentisate and the reactions analysed spectrophotometrically using overlay-spectra. This demonstrated that the cell extracts rather slowly converted homogentisate causing a batho- and hyperchromic shift. Homogentisate-1,2-dioxygenases are known to contain ferrous iron ions in their catalytical center (Adachi *et al*. 1966). Therefore, the cell extracts were incubated for 30 min with 2 mM $Fe(NH_4)_2(SO_4)_2$ prior to the enzyme assays. This resulted in a pronounced increase in the enzyme activity and it could be demonstrated that homogentisate was converted to a product with an absorption maximum at λ_{max}= 317 nm. This suggested that homogentisate was converted by a homogentisate-1,2-dioxygenase to maleylacetoacetate (Knox & Edwards 1955). The

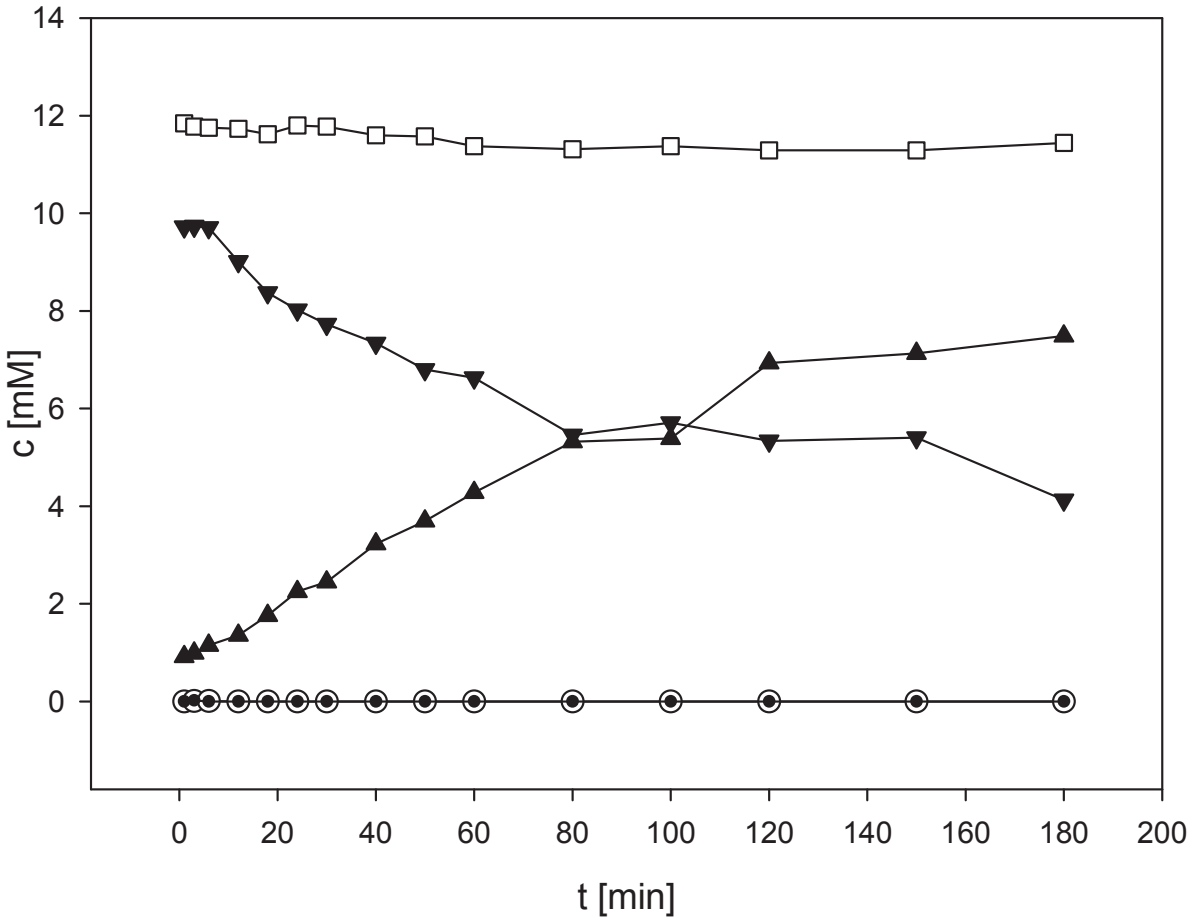

Fig. 4. Turn-over of 2-hydroxy-3-butenenitrile by resting cells of *E. oligosperma* R1 at pH 4. The resting cells were prepared as described in Fig. 3. The reaction mixture contained 0.1 M Na-citrate-phosphate-buffer (pH 4) and resting cells corresponding to an OD_{600nm} of 100. The control experiment contained 2 mL of the 0.1 M Na-citrate-phosphate-buffer (pH 4) without cells. The reaction mixtures were incubated in Eppendorf cups (2 mL at 30 °C in a thermoshaker (1 400 rpm). The reactions were started by the addition of 2-hydroxy-3-butenenitrile (10 mM). After different time intervals, samples (100 µL each) were taken, and the reactions terminated by the addition of 10 % (*v/v*) 1 M HCl. Cells were removed by centrifugation (21 000 g, 10 min, 4 °C) and the supernatants analysed by HPLC as described in the material and method section. The concentrations of 2-hydroxy-3-butenenitrile (▼), acroleine (●), and 2-hydroxy-3-butenoic acid (▲) in the experiment with cells as well as the concentrations of 2-hydroxy-3-butenenitrile (□) and acroleine (○) in the control experiment were calculated based on their signal intensities during the HPLC analysis.

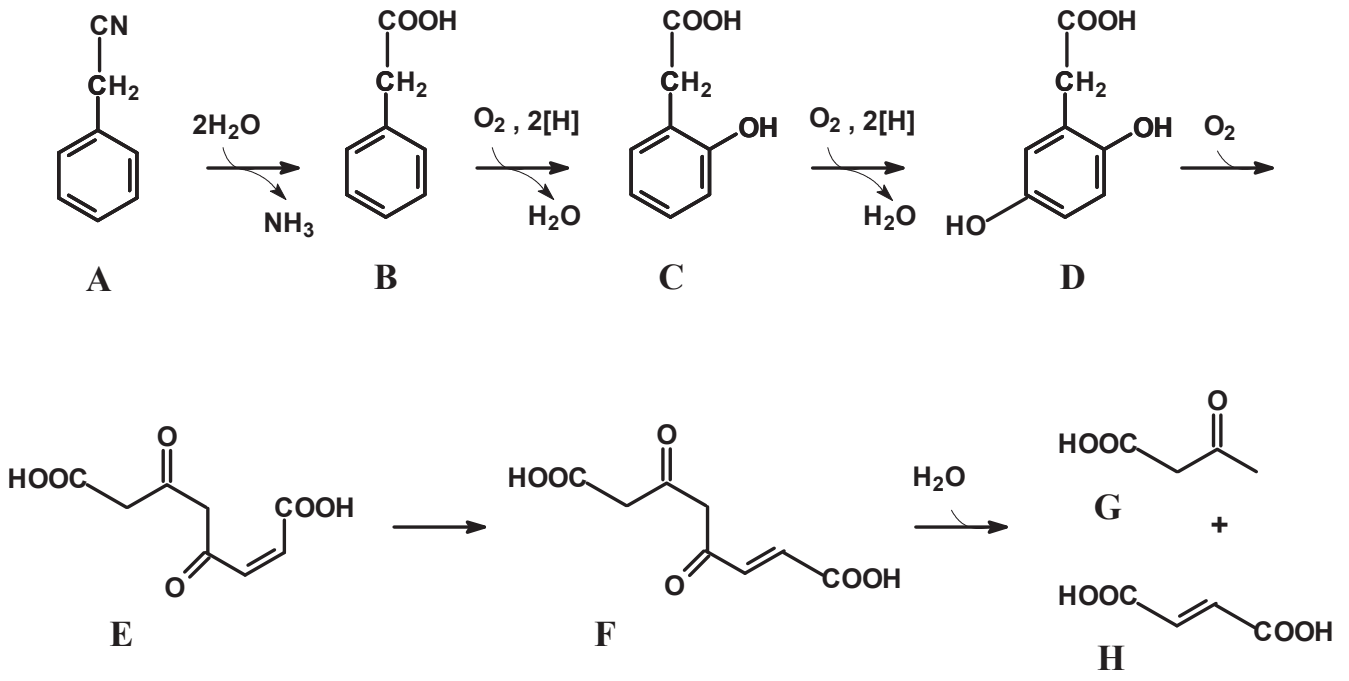

Fig. 5. Proposed pathway for the degradation of phenylacetonitrile by *E. oligosperma* R1. Key to compounds: A. phenylacetonitrile, B. phenylacetic acid, C. 2-hydroxyphenylacetic acid, D. 2,5-dihydroxyphenylacetic acid (homogentisic acid), E. maleylacetoacetic acid, F. fumarylacetoacetic acid, G. acetoacetic acid, H. fumaric acid.

specific activity was calculated from the known molar extinction coefficient of maleylacetoacetate (ε_{330nm}= 14 $mM^{-1}cm^{-1}$; Adachi *et al*. 1966) as 0.11 U mg^{-1} of protein. The reaction terminated when the initial concentration of homogentisate was converted and the calculated almost stoichiometric amounts of maleylacetoacetate were formed. The maleylacetoacetate was stable in the cuvettes for more than an hour. In most organisms maleylacetoacetate is converted by glutathione-dependent maleylacetoacetate isomerases (Fernánandez-Canón & Penalva 1998). Therefore, glutathione (2 mM) was added to the cuvettes and an immediate decrease in the absorbance at λ_{max}= 317 nm observed. This suggested that homogentisate is metabolised by *E. oligosperma* R1 via maleylacetoacetate and fumarylacetoacetate to fumarate and acetoacetate (Fig. 5). Thus, it appears that homogentisate is an important ring-fission substrate in "black yeasts", because it has already been suggested that *E. jeanselmei* (nowadays *Phialophora sessilis*) and *E. lecanii-corni* degrade compounds such as styrene and ethylbenzene via homogentisate (Cox *et al*. 1996; Gunsch *et al*. 2005; Prenafeta-Boldú *et al*. 2006).

DISCUSSION

The main aim of the present study was to obtain sufficient amounts of active biomass in order to allow a comparison of the nitrile converting system of *E. oligosperma* R1 with other (fungal) nitrile hydrolysing systems. The initial induction experiments demonstrated that the highest nitrile hydrolysing activities were achieved after growth of *E. oligosperma* R1 in the presence of 2-cyanopyridine. This compound had already been previously identified as a powerful inducer for the nitrile hydrolysing systems of different filamentous fungi such as *A. niger* K10, *Fusarium oxysporum* CCF1414, *F. oxysporum* CCF 483, *F. solani* O1, and *P. multicolor* CCF 244 (Kaplan *et al*. 2006b). 2-Cyanopyridine did not only serve as inducer of the nitrile converting activity of *E. oligosperma* R1 but was also metabolised and utilised as sole source of nitrogen as has been previously reported for *F. solani* O1 (Kaplan *et al*. 2006b).

The experiments with whole cells and cell extracts demonstrated that *E. oligosperma* R1 converted nitriles primarily to the corresponding acids and that the organism formed only from very few substrates low amounts of the corresponding amides. In addition, the cell extracts exhibited only a rudimentary amidase activity. Thus, it can be concluded that the nitriles were converted by a nitrilase. It therefore appears that most fungi convert organic nitriles using nitrilases and that nitrile hydratases are not common among fungi.

The extracts prepared from cells of *E. oligosperma* which had been grown in the presence of 2-cyanopyridine converted all available isomers of methyl-, hydroxy-, or chloro-substituted benzonitriles and phenylacetonitriles as well as phenylpropionitrile, 2-, 3- and 4-cyanopyridine. These cell extracts exhibited for these substrates compared to benzonitrile as substrate significantly higher relative activities than cell extracts or purified nitrilase fractions from *F. solani* O1, *P. multicolor* CCF 2244, or *A. niger* K10 (see Table 2). This was especially evident for the *meta*-substituted benzonitriles, because cell extracts of *E. oligosperma* R1 in general converted these substrates with higher specific activities than benzonitrile. In contrast, the opposite was reported for cell extracts from *F. solani* O1, *P. multicolor* CCF 2244, and the purified nitrilase of *A. niger* K10 (Kaplan *et al*. 2006a, c). The enzyme from *E. oligosperma* R1 also converted different *ortho*- substituted benzonitriles (*e.g.* 2-tolunitrile, 2-hydroxy- and 2-chlorobenzonitrile) which, probably due to sterical hindrances, could not be converted by the other strains (Kaplan *et al.* 2006a, c). In addition to the (substituted) benzonitrile(s) and phenylacetonitrile(s), the cell extracts from *E. oligosperma* R1 also converted aliphatic substrates such as acrylonitrile and 2-hydroxy-3-butenenitrile. The turn-over of acrylonitrile had not been analysed in the studies by Kaplan *et al*. (2006a, c) but had been previously described for another fungal nitrilase from *F. oxysporum* f. *sp melonis* (Goldlust & Bohak 1989). However, the enzyme of *F. oxysporum* f. *sp melonis* converted acrylonitrile (in comparison to benzonitrile as substrate) with significantly lower relative activities. Thus it can be concluded that the nitrile converting activity from *E. oligosperma* R1 appears to accept a slightly wider range of substrates than other fungal nitrilases.

The formation of amides as by-products of nitrilase catalyzed reactions had already been observed with various nitrilases from bacteria, plants, and fungi, *e.g.* from *Rhodococcus* ATCC 39484, *Pseudomonas fluorescens* EBC191, *Arabidopsis thaliana*, and *F. oxysporum* f. *sp. melonis*, and *A. niger* K10 (Goldlust & Bohak 1989, Stevenson *et al*. 1992, Effenberger & Osswald 2001, Osswald *et al*. 2002, Šnajdrová *et al.* 2004, Kiziak *et al*. 2005, Mateo *et al*. 2006, Fernandes *et al.* 2006, Kaplan *et al*. 2006b, c, Rustler *et al.* 2007). It was proposed that the formation of these by-products is based on an atypical cleavage of the tetrahedral intermediate formed during the reaction which might be triggered by electron withdrawing effects of different substituents (Fernandes e*t al*. 2006). The nitrilase activity from *E. oligosperma* R1 produced the largest relative amounts of amides during the induction experiments from 2-cyanopyridine (Fig. 2; ratio acid: amide about 7:4) and during the experiments with cell extracts (Table 2) from 2-chlorobenzonitrile (ratio acid: amide about 7:3). Furthermore some amides (< 10 molar % of total products) were also formed during the turn-over of 4-cyanopyridine and 3-chlorobenzonitrile. These results follow the emerging trend for the relationship between substrate structure and the degree of amide formation for fungal nitrilases. Thus, also *P. multicolor* CCF 2244 and the purified nitrilase from *A. niger* K10 produced relatively large amounts of amides from chlorobenzonitriles and cyanopyridines (Kaplan *et al*. 2006b, c). From the available data it appears that the nitrilase from *E. oligosperma* R1 is regarding to its tendency for amide formation situated somehow intermediate between the enzymes from *F. solani* O1 (less amide formation) and those from *P. multicolor* CCF 2244 and *A. niger* K10 (stronger tendency for amide formation).

In conclusion it might be summarised that the recent work in the group of L. Martinková (Kaplan *et al.* 2006a,b,c; Vejvoda *et al.* 2008) about the nitrile converting systems of filamentous fungi together with our study about the black yeast *E. oligosperma* R1 (Rustler & Stolz, 2007, this manuscript) suggest that evolutionary rather different fungi synthesise nitrilases which clearly resemble each other regarding their induction system and substrate specificity. These fungal systems might be of some relevance because of the high specific activities that can be obtained under optimal induction conditions and also the high specific activities of the purified enzymes with their preferred substrates. In addition, it was shown in the present study that the acid tolerance of fungi allows their utilisation as whole cell catalysts for the conversion of nitriles that are stabilised under acidic conditions. This observation might significantly enhance the importance of fungal nitrilases for biotransformation reactions.

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