Appl Microbiol Biotechnol (2005) 68: 327–335
DOI 10.1007/s00253-005-1903-8

Dakshina M. Jandhyala · Richard C. Willson · B. Trevor Sewell · Michael J. Benedik

Comparison of cyanide-degrading nitrilases

Received: 23 September 2004 / Revised: 21 December 2004 / Accepted: 22 December 2004 / Published online: 10 February 2005

Abstract Recombinant forms of three cyanide-degrading nitrilases, CynD from *Bacillus pumilus* C1, CynD from *Pseudomonas stutzeri*, and CHT from *Gloeocercospora sorghi*, were prepared after their genes were cloned with C-terminal hexahistidine purification tags and expressed in *Escherichia coli*, and the enzymes purified using nickel-chelate affinity chromatography. The enzymes were compared with respect to their pH stability, thermostability, metal tolerance, and kinetic constants. The two bacterial genes, both cyanide dihydratases, were similar with respect to pH range, retaining greater than 50% activity between pH 5.2 and pH 8 and kinetic properties, having similar K_m (6–7 mM) and V_{max} (0.1 mmol min^{-1} mg^{-1}). They also exhibited similar metal tolerances. However, the fungal CHT enzyme had notably higher K_m (90 mM) and V_{max} (4 mmol min^{-1} mg^{-1}) values. Its pH range was slightly more alkaline (retaining nearly full activity above 8.5), but exhibited a lower thermal tolerance. CHT was less sensitive to Hg^{2+} and more sensitive to Pb^{2+} than the CynD enzymes. These data describe, in part, the current limits that exist for using nitrilases as agents in the bioremediation of cyanide-containing waste effluent, and may help serve to determine where and under what conditions these nitrilases may be applied.

D. M. Jandhyala · R. C. Willson · M. J. Benedik
Department of Biology and Biochemistry,
University of Houston,
Houston, TX 77204-5001, USA

Present address:
D. M. Jandhyala
Institute of Veterinary, Animal and Biomedical Sciences,
Massey University,
Private Bag 11 222,
Palmerston North, New Zealand

R. C. Willson
Department of Chemical Engineering,
University of Houston,
Houston, TX 77204-5001, USA

B. T. Sewell
Electron Microscope Unit,
University of Cape Town,
Rondebosch 7701,
Cape Town, South Africa

Present address:
M. J. Benedik (✉)
Department of Biology, Texas A&M University,
College Station, TX 77843-3258, USA
e-mail: benedik@tamu.edu
Tel.: +1-979-8455776
Fax: +1-979-8452891

Introduction

Cyanide is used in a variety of industrial processes including electroplating, plastics manufacturing, and extraction of gold from ore. These industrial processes generate large quantities of cyanide-containing waste that must be detoxified prior to being released into the environment. The inconvenience and cost of conventional cyanide detoxification methods creates a need for simpler and more economical methods. A variety of organisms including plants, animals, fungi, and bacteria can convert cyanide to non-toxic byproducts, and biological solutions for cyanide detoxification have been investigated and even in some cases, applied with moderate success. These methodologies have involved using viable cyanide-degrading organisms either as pure cultures or as multispecies consortia applied directly to a given waste effluent (Basheer et al. 1993; Mudder and Whitlock 1984), or using crude lysates of a cyanide-degrading organism have been developed. However, few reports have considered the use of purified enzyme for the detoxification of cyanide-containing waste.

As there are varieties of organisms that are able to metabolize cyanide, likewise there are a variety of enzymes that have been shown to convert cyanide to non-toxic products. The cyanide-degrading nitrilases differ from many of those enzymes in that they are able to hydrolyze cyanide without the addition of any cofactors or secondary substrates.

The cyanide-degrading nitrilases include the related fungal cyanide hydratases of *Fusarium lateritium*, *Gloeocercospora sorghi*, *Leptosphaeria maculans*, and the bac-

terial cyanide dihydratases of *Alcaligenes xylosoxidans* subsp. *denitrificans*, *Bacillus pumilus* and *Pseudomonas stutzeri* AK61, all of which efficiently hydrolyze cyanide to either formamide (fungal cyanide hydratase, CHT) or formate and ammonia (bacterial cyanide dihydratase, CynD) (Nolan et al. 2003; Cluness et al. 1993; Fry and Munch 1975; Ingvorsen et al. 1991; Meyers et al. 1993; Sexton and Howlett 2000; Wang et al. 1992; Watanabe et al. 1998a). These enzymes have been investigated for use in industrial cyanide remediation and the organisms *F. lateritium* and *A. xylosoxidans* subsp. *denitrificans* have been patented for use as cyanide-remediating biocatalysts (Ingvorsen et al. 1992; Richardson and Clarke, 1993). However, the lack of comparative studies, structural analysis, and use of recombinant DNA technologies leaves much room for optimizing the application of these enzymes to the clearance of cyanide from industrial wastes.

In deciding upon a strategy for cyanide removal, many physical and chemical factors need to be considered. The many different uses for cyanide result in the production of wastewaters that vary in qualities such as pH and solute content. Therefore, it is necessary to understand the physical and chemical limitations of these enzymes. This work represents the first direct comparison of stabilities and kinetics among three recombinant cyanide-degrading nitrilases, the cyanide hydratase of *G. sorghi*, the cyanide dihydratase of *B. pumilus* C1, and the cyanide dihydratase of *P. stutzeri* AK61.

Materials and methods

Bacterial strains and plasmids

The plasmids and strains used in this study are described in Table 1.

Culture media and reagents

Unless otherwise mentioned, all strains were grown on Luria broth. Antibiotics were added to concentrations of 100 μg/ml ampicillin, 30 μg/ml chloramphenicol, and 25 μg/ml kanamycin, for selection in *E. coli* strains. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was used at a concentration of 20 μg/ml.

Genomic and plasmid DNA preparation

Genomic DNA from *P. stutzeri* AK61 was prepared using the Bactozol Kit (Molecular Research Center, Cincinnati,

Table 1 Bacterial strains and plasmids

Strains and plasmids	Description/genotype	Reference
Escherichia coli		
BL21(DE3), pLysS	F⁻ *ompT hsdS*$_B$*gal dcm* (DE3)	Novagen
MB1547	*SupE thi hsd*Δ5 (Δ*lac-proAB*) Δ*endA*/*F′* [*traD*36 *proAB lacI*^{q}*lacZ*Δ*M*15]	Laboratory stock
MB2254	MB1547 pPW1	This work
MB2313	BL-21 (DE3) pLysS p2313	This work
MB2333	BL-21 (DE3) pLysS p2333	This work
MB2784	BL-21 (DE3) pLysS p2784	This work
MB2785	BL-21 (DE3) pLysS p2785	This work
MB2890	BL-21 (DE3) pLysS p2890	(Jandhyala et al. 2003)
MB2899	BL-21 (DE3) pLysS p2899	(Jandhyala et al. 2003)
MB3033	BL21 (DE3) pLysS p3033	(Jandhyala et al. 2003)
Pseudomonas stutzeri		
AK61	Cyanide dihydratase producing strain of *P. stutzeri*	(Watanabe et al. 1998a)
Plasmids		
pET-26b	Kan^R*E. coli* expression vector	Novagen
pPW1	pBS (KS-) with a 3.1-kb genomic DNA insert from *Gloeocercospora sorghi* containing the *cht* gene	(Wang and VanEtten 1992)
P2160	pBS(KS+)-derived Ap^R*E. coli* cloning vector with an *Nde*I site replacing the *Cla*I site	Lab stock
P2313	pET26b *Nde*I–*Xho*I fragment of *G. sorghi cht*	This work
P2333	pET26b *Nde*I–*Xho*I fragment of *G. sorghi cht*-6His	This work
P2784	pET26b *Nde*I–*Xho*I fragment of *P. stutzeri* AK61 *cynD*$_{stut}$[a]	This work
P2785	pET26b *Nde*I–*Xho*I fragment of *P. stutzeri* AK61 *cynD*$_{stut}$-6His[a]	This work
P2899	pET26b *cynD*$_{pum}$-6His[a] as a *Nde*I–*Xho*I fragment	(Jandhyala et al. 2003)
P3177	p2160 *cynD*$_{stut}$ PCR cloned in the *EcoRV* site	This work

[a]The subscript *pum* or *stut* following *cyn*D (e.g. *cyn*D$_{pum}$) refers to the origin of the corresponding cyanide dihydratase gene as cloned from *Bacillus pumilus* C1 (pum) or *P. stutzeri* AK61 (stut)

Ohio). Plasmid DNA from *E. coli* strains was prepared using the Wizard Plus Miniprep DNA Purification System (Promega, Madison, Wis.).

PCR methodology

Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen Life Technologies, Carlsbad, Calif.) was used for all PCR reactions with the following conditions: annealing time was kept at 30 s at the specified temperature, elongation time was 1 min at 68°C, and denaturation was 30 s at 96°C. A general two-step PCR method was employed for the PCR amplification of the cyanide dihydratase gene from genomic DNA of *P. stutzeri* AK61, using oligonucleotide primers Stutz-N1 and Stutz-C1 (Table 2). This method consisted of ten cycles, with an annealing temperature of 50°C, followed by 25 cycles with an annealing temperature of 58°C. For amplification of this gene from plasmid DNA, using primers Stutz-NdeI along with either Stutz-XhoI or Stutz-His (Table 2), a one-step method consisting of 20 cycles at 58°C was used. A two-step PCR method consisting of ten cycles with an annealing temperature of 55°C and 30 cycles with an annealing temperature of 62°C was employed for amplification of *cht* from pPW1.

Expression and purification of CynD and CHT from *E. coli*

Starter cultures of MB2333, MB2785, or MB2899 were grown at 30°C and 250 rpm overnight. Fresh 1-l cultures were started using 1/100 volume of an overnight culture and incubated in 2-l flasks at 30 or 37°C, with shaking until the OD_{600} approached 0.5. The cells were induced by the addition of IPTG to a final concentration of 1 mM and were further incubated for 3–10 h at 30°C with shaking prior to being harvested.

Cell pellets were resuspended in 20 mM phosphate buffer-100 mM NaCl (pH 7.8) plus 10 mM imidazole (pH 7.8) and lysed by disruption with a French press three to four times at 1,000 psi. The insoluble material was removed by centrifugation at 10,000 *g* for 10 min.

For small-scale preparations, cell pellets from 1 ml of induced MB2890, MB2899, or MB3033 cultures were resuspended in 500 μl 200 mM MOPS, 200 mM NaCl (pH 7.6). The resuspended pellet was then incubated at 37°C for 20 min with 2 mg ml^{-1} lysozyme. Deoxycholate was added to 0.1% and the samples kept on ice for 15 min. The soluble fraction was separated from the insoluble material in a microcentrifuge.

The hexahistidine (6-His)-tagged cyanide dihydratase enzyme was purified from crude cell lysates, using immobilized nickel-ion affinity chromatography employing a 5-ml HiTrap metal chelating column (Amersham Pharmacia Biotech, Piscataway, N.J.) charged with 2.5 ml 0.1 M $NiSO_4$. After passing the soluble lysate through the charged column, it was washed one to three times with ten column volumes of Start buffer consisting of 20 mM sodium phosphate (pH 7.8), 100 mM NaCl, and 10 mM imidazole, followed by ten column volumes Start buffer + 100 mM imidazole. Bound enzyme was eluted in Start buffer + 500 mM imidazole. Fractions having detectable cyanide-degrading activity were pooled and dialyzed against 50 mM MOPS-100 mM NaCl (pH 7.8) for CynD, or 50 mM MOPS (pH 6.9)-100 mM NaCl-100 mM imidazole and 5% v/v glycerol for CHT. For storage at −80°C, the glycerol concentration was increased to 30% for all enzymes.

Table 2 Primer sequences. The primer sequences are written in 5′-to-3′ direction. The Xho primers introduce an *Xho*I site just after the stop codon. The His primers introduce a *Xho*I site just before the stop codon so as to create an in-frame fusion to the His-tag sequence of pET26b

Primer	Sequence
Stutz-N1	ATG GCG CAT TAC CCT AAA TTC AAG GCT GCG GC
Stutz-C1	ACT TCC GCA AGC TCC GAA CGA CTG GCT CTT C
Stut-NdeI	GAT ACA TAT GGC GCA TTA CCC TAA ATT CAA GGC T
Stut-XhoI	CAC ACT CGA GTT TAC TTC CGC AAG CTC CGA ACG ACT
Stut-His	CAC ACT CGA GCT TCC GCA AGC TCC GAA CGA CTG G
Gsorgh-NdeI	GAG AGA TAC ATA TGC CCA TCA ACA AGT ACA AGG CG
Gsorgh-XhoI	CAC ACT CGA GTT TAG GCA TGT CCA TTG GAA CTA GC
Gsorgh-His	CAC ACT CGA GGG CAT GTC CAT TGG AAC TAG CTT

pH activity profiles

The pH activity profiles for the recombinant purified cyanide-degrading nitrilases of *G. sorghi*, *P. stutzeri* AK61, and *B. pumilus* C1 were determined using MOPS buffer from pH 6.2 to pH 8.4 and sodium acetate between pH 4.9 and pH 7.1. For each pH condition, a 1-M stock solution of KCN was made in 10 ml of 1-M buffer. Just before use, the KCN was diluted another 50-fold to yield a final KCN concentration of 20 mM and a final buffer concentration of 0.5 M. Reactions were run at 37°C in a final volume of 1 ml consisting of enzyme diluted in H_2O (0.9 ml) and buffered KCN (0.1 ml), both pre-warmed for 15 min at 37°C. Enzyme concentrations used for rate measurements were 5.5 μg ml^{-1} for $CynD_{pum}$ and $CynD_{stut}$, and 1.6 μg ml^{-1} for CHT. Reactions were run for 6 min, with samples taken at time points of 1 min and 6 min. Using the picric acid endpoint method for determining cyanide concentration (Fisher and Brown 1952), the rate of cyanide degradation

was measured as the change in OD_{520} per minute during this 5-min interval. Pilot experiments within the optimal pH range were run with samples taken at 1-min time points to demonstrate that the rates over the 5-min time course were indeed linear.

Measurement of heavy metal effects

To determine the sensitivity of the different enzymes to the presence of various metals, each of the three enzymes were assayed for cyanide degradation in the presence of 200 μM $CrCl_3$, $FeCl_3$, $CdCl_2$, $HgCl_2$, $PbCl_2$, $ZnCl_2$, $HAuCl_4$, $NiSO_4$, $CuSO_4$, and $AgNO_3$. The 1-ml assay reactions contained 200 μM of the metal salt, 50 mM MOPS, 2 mM KCN made using a 1-M stock solution of MOPS pH 7.6, and were assayed at 37°C, using 3.9, 3.9, and 1.2 μg ml^{-1} of $CynD_{pum}$, $CynD_{stut}$, and CHT, respectively. Samples were taken at 1 min and 6 min, following the addition of enzyme to measure the remaining cyanide. Prior to testing, the different heavy metals against a given enzyme, pilot reactions with no added metals were run taking 1 min CN^- measurements to verify linearity between the 1-min and 6-min time points.

Determination of temperature optima and thermal stability

To determine the optimal temperature for cyanide degradation by each of the three enzymes, the rate of cyanide hydrolysis was measured at temperatures of 23, 30, 37, and 42°C. Reaction conditions were the same as used for above for assaying metal effects.

To determine the thermal stability of the enzymes, each enzyme was incubated at temperatures of 23, 37, 42, and 55°C for time periods between 30 min and 30 h, following which, the activities were measured at 23°C over a time course of 8 min, as described above.

Kinetic measurements

Initial rates were measured at 23°C for each enzyme, using substrate concentrations above and below the reported K_m for wild-type enzymes (Meyers et al. 1993; Wang et al. 1992; Watanabe et al. 1998b). A 1-M buffered cyanide stock solution was made in 1 M MOPS. For use, this was diluted to maintain 50 mM MOPS in the reaction, regardless of KCN concentration. Initial rates were measured at 0.5-min and/or 1-min time points to determine whether departure from linearity was occurring within the 5 min of the reaction. Enzyme concentrations of 3.9, 3.9, and 2.3 μg ml^{-1} of enzyme were used for kinetic analysis of $CynD_{pum}$, $CynD_{stut}$, and CHT respectively.

Results

Cloning of *cynD* of *P. stutzeri* AK61 and *cht* of *G. sorghi* for expression in *E. coli*

The *cynD* gene was PCR-amplified from *P. stutzeri* AK61 (IAM No. 14761) genomic DNA, using the primers Stutz-N1 and Stutz-C1 (Table 2), treated with T4 polymerase, and ligated to the *Eco*RV site of p2160 to make p3177. Using this plasmid as a template, the primers sets (1) Stut-NdeI and Stut-XhoI, and (2) Stut-NdeI and Stut-His were used to reamplify *cynD* to confer restriction sites *Nde*I and *Xho*I for cloning into pET26b, with (Stut-His) and without (Stut-XhoI) a translational C-terminal 6-His fusion to make strains MB2784 and MB2785.

Cht was amplified from pPW1 (Table 1) ,using the two primer sets Gsorgh-NdeI with Gsorgh-XhoI, or Gsorgh-NdeI with Gsorgh-His (Table 2). These were ligated to pET26b and transformed into BL-21 (DE3) pLysS to make strains MB2313 and MB2333. All four strains expressed cyanide-degrading activity after induction with IPTG, unlike the control. Overexpression of CynD and CHT was readily detectable on SDS-PAGE when induced cell lysates were compared to uninduced cell lysates (data not shown). As there was not a noticeable difference in activity with the presence or absence of the His tag, all subsequent studies in this work utilize the affinity purified His-tagged product described below.

Affinity purification of recombinant CynD and CHT enzymes

$CynD_{pum}$, $CynD_{stut}$, and CHT were purified from MB2899, MB2785, and MB2333, respectively (Table 1), using nickel-resin affinity chromatography as described, and SDS-PAGE was used to analyze the purity of the protein preparation. Of the three enzymes, the hydratase CHT proved somewhat problematic, as precipitate readily formed during the purification procedure. Wang et al. (1992) reported that following 5 h of centrifugation at 120,000 *g*, all CHT activity in cell-free extracts of *G. sorghi* was found to be in the precipitate. We determined that solubility of the eluted protein was maintained by dialyzing His-purified eluate against 50 mM MOPS (pH 6.9), 100 mM NaCl, and 100 mM imidazole.

pH tolerance of CynD and CHT enzymes

The pH activity profile of each of the affinity purified $CynD_{pum}$, $CynD_{stut}$, and CHT enzymes was measured (Fig. 1). All three displayed activity maxima in the range of pH 7–8, with a fall-off in activity at both low and high pH. The activity of each of the cyanide dihydratase enzymes decreased abruptly as the pH rose above 7.8, dis-

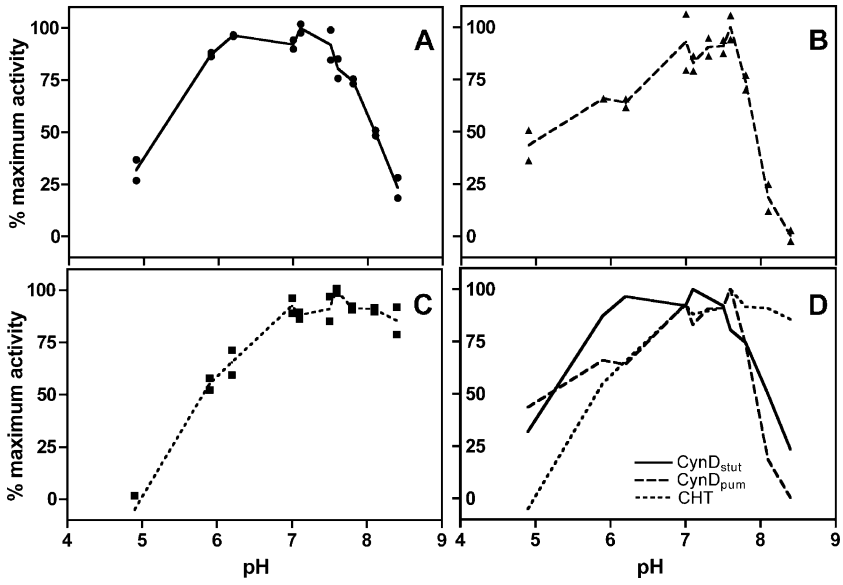

Fig. 1 The pH activity profiles of **a** $CynD_{stut}$ from MB2785, **b** $CynD_{pum}$ from MB2899, and **c** CHT from MB2333 (respectively). **d** Overlapping profiles of all three enzymes. *Symbols* represent values from duplicate experiments with the *line* as the average

playing 50% activity at approximately pH 8 in both cases. The activity curve for CHT was shifted to the right, and little decrease in activity was measured up to pH 8.4. A decrease in activity was also seen at low pH, with both CynD enzymes displaying 50% activity at approximately pH 5.2, and CHT displaying 50% activity at pH 5.8. Interestingly, the nature of the decrease in activity at low pH is completely different in the case of $CynD_{pum}$. Whereas the activities of both CHT and $CynD_{stut}$ decrease monotonically as a function of decreasing pH below 7 with approximately the same slope, the activity of $CynD_{pum}$ increases between pH 6.2 and 5.8.

Tolerance to heavy metals

To evaluate the sensitivity of these enzymes to heavy metals, cyanide degradation was measured separately in the presence of ten metals. All experiments (shown in Fig. 2) were performed using a single-substrate concentration of 2 mM cyanide. The studies of four of the metals Cu^{2+}, Ni^{2+}, surable cyanide concentration appeared to decrease in the presence of these metals, whether due to interference with the assay or by the formation of complexes is unknown. It therefore was not possible to accurately determine their effect on enzyme inhibition, but in no case was it greater than 50%, based on the remaining measurable cyanide. The other metals, Cd^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, Cr^{3+}, and Fe^{3+}, had a far smaller, if any, effect on the measured cyanide concentration, and therefore changes in enzyme activity could be ascertained.

The initial rates of all three enzymes were appreciably depressed in the presence of Hg^{2+}. In addition, 200 μM Pb^{2+} appeared to somewhat inhibit each of the three enzymes, with the greatest effect being on CHT. Zn^{2+} and Cd^{2+} showed only slight inhibition of all three enzymes.

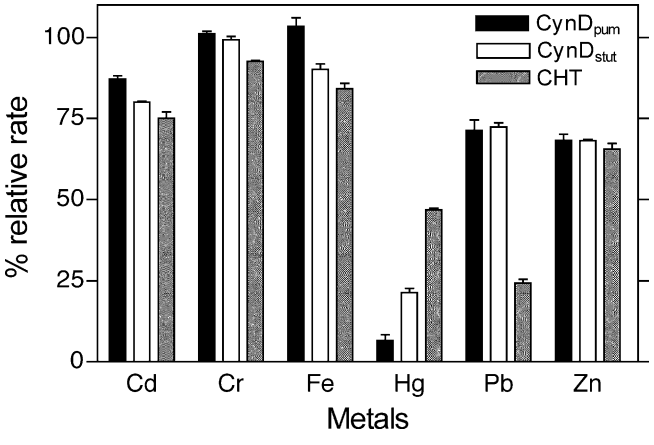

Fig. 2 Comparison of the relative rates of enzyme activity in the presence of 200 μM of Hg^{2+}, Pb^{2+}, Cd^{2+}, Zn^{2+}, Cr^{3+}, and Fe^{3+} for the enzymes $CynD_{pum}$, $CynD_{stut}$, and CHT. The *y-axis* is the percent activity relative to that of the enzyme with 2 mM KCN in the absence of added metal. The *error bars* represent the standard deviation (SD) (n=3)

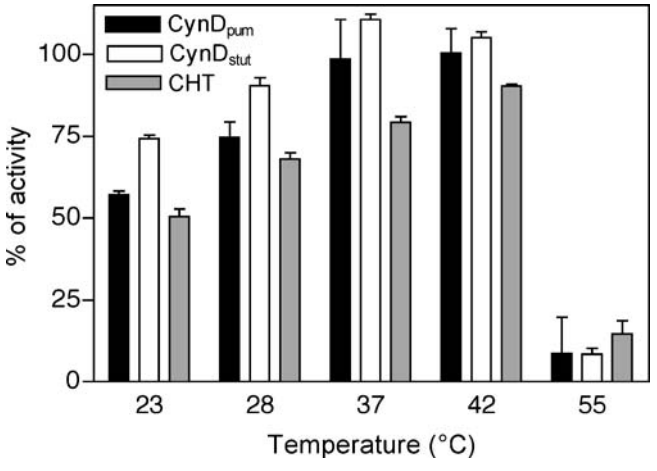

Fig. 3 Relative activities were taken at five different reaction temperatures to determine the optimal temperature range for cyanide degradation. Relative activities were calculated with respect to the activity at optimal temperature. The *error bars* represent the SD (n=3)

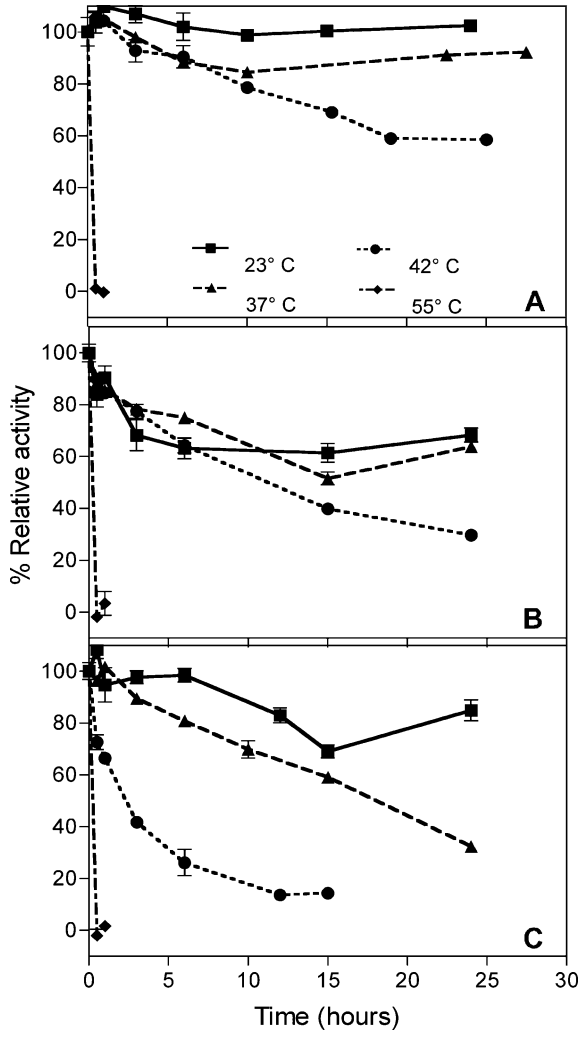

Fig. 4 The thermal stabilities of **a** $CynD_{pum}$, **b** $CynD_{stut}$, and **c** CHT. Residual activities after incubation at the noted temperatures for periods between 0 h and 27.5 h. *Error bars* represent the SD (n=3)

Temperature stability and operating range

Previous reports had demonstrated that the temperature optima of CynD from *B. pumilus* C1, *P. stutzeri* AK61, and CHT from *G. sorghi* were 37, 30, and 37°C respectively (Fry and Munch 1975; Meyers et al. 1993; Watanabe et al. 1998a). To compare directly the operating temperature range of the recombinant enzymes, reactions were run at 23, 28, 37, 42 and 55°C (Fig. 3). Both CynD enzymes appeared to have maximum activity between 37°C and 42°C, while CHT reaches maximal activity between 42°C and 55°C.

To determine how stable the enzymes were at various temperatures, each enzyme was incubated at 23, 37, 42, and 55°C for time periods ranging between 0 h and 30 h, following which their relative residual activities were measured (Fig. 4). $CynD_{pum}$ (Fig. 4a) was most stable, losing no activity when incubated at 23°C and maintaining over 50% activity when incubated at 42°C for over 24 h. $CynD_{stut}$ (Fig. 4b) appeared to quickly lose activity even at 23°C and 37°C; however, following the initial loss of activity (≈30–40% in 6 h), the remaining activity was stable over the following 18 h of incubation. CHT appears to be the least thermostable of the three enzymes (Fig. 4c). Although little activity was lost after 24 h of incubation at 23°C, approximately 70% of the activity was lost at 37°C compared with less than 20% and 40% for $CynD_{pum}$ and $CynD_{stut}$ respectively. CHT also appeared to lose activity at 42°C, much faster than the other two enzymes. All enzymes were inactivated completely by incubation at 55°C for 30 min.

Kinetic analysis

Kinetic analyses were performed on all three enzymes at room temperature (23°C). A total of five points were taken for each enzyme, including two substrate concentrations above and below the K_m previously reported for each of the wild-type CynD and CHT enzymes. Table 3 lists the determined K_m and V_{max} values of the recombinant His-tagged enzymes as well as the values previously reported for the wild-type enzymes.

The K_m values determined for the three enzymes were all higher than those reported for the corresponding wild-type enzymes (Table 3). It is not apparent whether this is due to the addition of the His tag or if this results from procedural differences in the analyses. However, kinetics experiments performed on the non-His-tagged $CynD_{pum}$ (data not shown) yielded results similar to those of the His-tagged enzymes, suggesting that procedural differences are probably responsible for the apparent differences. Both CynD enzymes have similar K_m and V_{max} values, while CHT has K_m and V_{max} values circa tenfold that of the CynD enzymes. While the standard error of the mean for these experiments is relatively high, it is clear that CHT has the highest activity of the group.

Table 3 Kinetic parameters

Enzyme	K_m (mM)	pH/temperature (°C)	V_{max} (mmol min^{-1} mg^{-1})	Source
$CynD_{pum}$[a]	7.3±1.4	7.6/23	0.097±0.011	This work
$CynD_{stut}$[a]	5.9±2.0	7.6/23	0.10±0.016	This work
CHT[a]	90±35	7.8/23	4.4±1.5	This work
$CynD_{pum}$[b]	2.56±0.48	8.0/37	0.088±0.004	(Meyers et al. 1993)
$CynD_{stut}$[b]	2.8	7.6/30	Not available	(Watanabe et al. 1998b)
CHT[b]	12	8.0/25	Not available	(Wang et al. 1992)

[a]Enzymes generated in this work are hexahistidine-tagged
[b]These data are from published sources and placed here for comparison

Discussion

Like *cynD* of *B. pumilus* C1, the genes *cynD* and *cht* of *P. stutzeri*, and *G. sorghi*, respectively, were actively expressed in *E. coli* with and without 6-His purification tags, which had no noticeable effect on activity. The tagged enzymes were used for comparison with respect to their kinetic properties, inhibition by metals, pH, and temperature ranges and stability.

pH profiling of the three recombinant enzymes demonstrates that CHT (Fig. 1) is more tolerant of higher pH values compared to the two CynD enzymes (greater than 80% relative activity at pH 8.4, whereas the CynD enzymes rapidly lose activity above this pH). A discussion of the

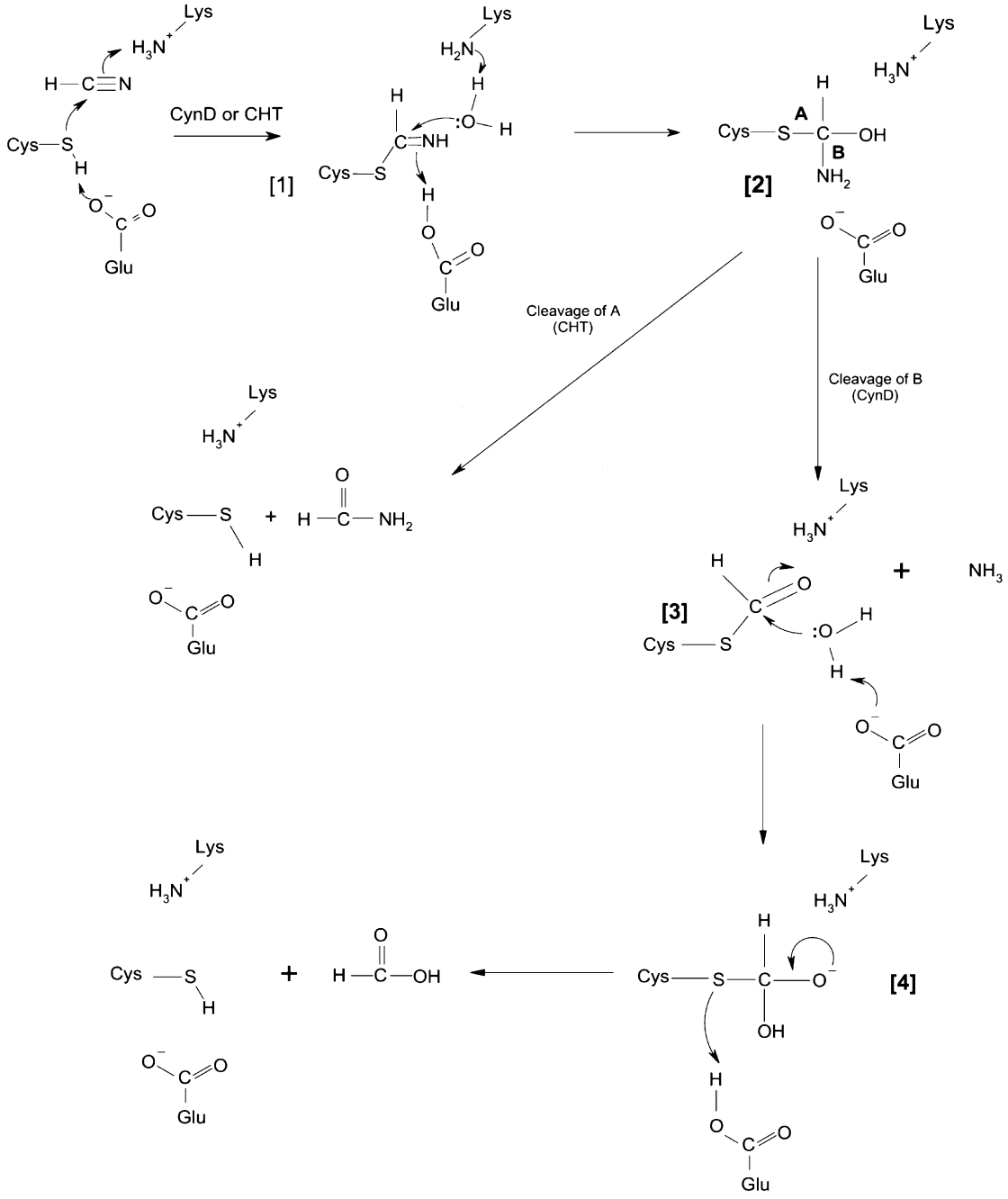

Fig. 5 The proposed mechanism of the cyanide hydratases and dihydratases. Glutamate, acting as a general base, activates the nucleophilic attack by the cysteine on the nitrile forming a thioimidate (*[1]*). The lysine, acting as a general base, then catalyzes a hydrolysis, leading to the tetrahedral intermediate (*[2]*). In the cyanide hydratases the C–S bond breaks leading to the release of formamide, whereas in the cyanide dihydratases the C–N bond breaks leading to the release of ammonia and the formation of the acylenzyme (*[3]*). Glutamate then activates a second hydrolysis leading to a second tetrahedral intermediate (*[4]*), which decomposes to release formic acid

pH dependence is facilitated by reference to the possible reaction mechanism shown (Fig. 5). Brenner (2002) has reviewed the evidence that the key residues involved are a glutamic acid, cysteine, and lysine. We propose roles for the lysine and glutamic acid that are similar to those proposed by Nakai et al. (2000) in the case of the *N*-carbamyl-D-amino acid amidohydrolase. The covalent intermediates depicted (Fig. 5) were initially suggested by Kobayashi et al. (1992), and the evidence for them has been recently reviewed by O'Reilly and Turner (2003). The pK_as of the reactive groups in step 1 are typically listed as 4.07 for glutamic acid, 8.33 for cysteine, 9.1 for HCN and 10.79 for lysine. It is well known that pK_as of side groups in enzyme active sites can vary considerably from the typical values because of their interactions with neighboring residues. The general form of the observed pH dependence is therefore not surprising, as one would expect the glutamic acid in the active site to be protonated at low pH, thus rendering it incapable of removing the proton from the catalytic cysteine residue, whereas at high pH the CN^- ion is incapable of accepting an electron. In light of this, the differences in the pH-dependent behavior of the three enzymes are clearly interesting, but further studies of the three dimensional amino acid context are necessary to explain the details.

The behavior of $CynD_{pum}$ is particularly interesting, as it does not decrease monotonically as the pH decreases (as the other two do). This suggests an additional process, other than titration of the Glu, is at work in this enzyme at pH 6, where in fact, a small increase in activity can be observed. It is well established that the three cyanide degrading nitrilases form large multimers. They differ, however, in the number of subunits per multimer, $CynD_{stut}$ being a 14mer (Sewell et al. 2003), $CynD_{pum}$ an 18mer at pH 8 (Jandhyala et al. 2003), and CHT yet to be accurately determined but believed to be greater than 300 kDa and able to form aggregates greater than 10 MDa (Fry and Munch 1975; Wang et al. 1992). Negative stain electron microscopy studies of $CynD_{pum}$ has shown that it undergoes a structural change from small discrete spirals approximately 18.9 nm in length to rods greater than 100 nm when the pH is decreased gradually below 6.0 (Jandhyala et al. 2003). There is, in fact, a precedent amongst other nitrilases for a correlation between enzyme activity and multimerization. For example, the nitrilases from *Rhodococcus rhodochrous* J1 and NCIB11216 both undergo an increase in activity towards certain substrates corresponding with the formation of active decameric and dodecameric complexes (Nagasawa et al. 1990; Harper 1977). This leads us to speculate that for $CynD_{pum}$ the slight increase in activity around pH 6 results from a small number of subunits becoming activated. If the terminal subunits in the helices are inactive, then the structural transformation to a long spiral would result in fewer end subunits and hence, more active subunits in the population.

Kobayashi et al. (1998) demonstrated that the nitrilase from *R. rhodochrous* J1 had slight amidase activity, and that both the nitrilase and amidase activities were terminated by mutations in the active site cysteine 165. In addition, the nitrilases from *Pseudomonas* (Hook and Robinson 1964), *Fusarium* (Goldlust and Bohak 1989), and *Rhodococcus* ATCC39484 (Stevenson et al. 1992) are able to produce small amounts of amide product in addition to the characteristic nitrilase products of ammonia and corresponding carboxylic acid. Kobayashi et al. (1998) therefore proposed a mechanism that suggests a similarly conserved enzyme-thioimidate intermediate is responsible for both the classic nitrilase activity as well as the amide producing activity seen in the cyanide hydratases. It is possible that the conversion to an amide product through the cleavage of the C–S bond and the stability to high pH (presumably through the stabilization of HCN in the active site) are correlated. In fact, *Fusarium solani* was shown to degrade cyanide via cyanide hydratase at a pH as high as 10.7 (Dumestre et al. 1997). It will be interesting to see whether the other cyanide hydratases are also tolerant to high pH, and whether the ancillary amide producing activity of the aforementioned nitrilases is favored by increasing the pH.

As might be expected, the effect of heavy metal on the activity of these enzymes depends upon the species. Hg^{2+}, added as $HgCl_2$, had the greatest effect and reduced the activity of all three enzymes. In addition, Pb^{2+} showed an appreciable effect on the activity of the CHT enzyme, but it was much less inhibitory on the CynD enzymes. Zn^{2+} and Cd^{2+} also showed slight inhibition of all three enzymes. In previous studies, Zn^{2+} was shown to have no effect on wild-type CynD of either *P. stutzeri* or *B. pumilus*, but it was reported that 1 mM Zn^{2+} caused over 52% inhibition of wild-type CHT (Meyers et al. 1993; Wang et al. 1992; Watanabe et al. 1998b). Our study of the His-tagged versions of these enzymes demonstrates that Zn^{2+} does have a slight inhibitory effect on the three His-tagged enzymes. We should note that we have not factored in the formation of complexes between CN and the added metals, which may alter the amount of available metal ions. However, we believe this is only significant for Ni^{2+} and Fe^{3+}.

That Hg^{2+} and Pb^{2+} inhibit all three enzymes is not unexpected as one of the key active site residues is believed to be a cysteine, and inhibition of an enzyme by Hg^{2+} and Pb^{2+} often implies that a sulfhydryl moiety is involved in catalysis. In fact, mutagenesis experiments have shown that all activity is lost in the *F. lateritium* (cyanide hydratase) and *P. stutzeri* (cyanide dihydratase) enzymes when site-directed mutations are made to cysteines 163 and 170, respectively (Brown et al. 1995; Watanabe et al. 1998c). In addition, experiments testing CHT in the presence of the sulfhydryl inhibitors Hg-acetate and *p*-chloromercuric benzoate at concentrations of 1 mM resulted in 100% inhibition of CHT activity (Wang et al. 1992). Based on the rate analysis, it appears that the CynD enzymes are more sensitive to Hg^{2+} and less sensitive to Pb^{2+}, while the opposite is true for CHT.

In general, the most pronounced differences were observed between the CynD enzymes and CHT. These including a tolerance to higher pH by CHT, a different sensitivity profile to Hg^{2+} and Pb^{2+}, and far larger K_m and V_{max} values by CHT. These differences are not surprising. The two CynD enzymes carry out the same reaction and are

over 80% identical at the amino acid level (excluding the highly divergent C-terminal tail), whereas the CHT enzyme catalyzes a slightly different reaction and is only weakly homologous (<30% identity) to the CynD enzymes.

Through further structural studies and application of site-directed mutagenesis or by directed evolution, more stable, or metallo-cyanide degrading nitrilases may be engineered. The discovery of a cyanide hydratase from *F. oxysporum* that has the ability to hydrolyze the nickel cyanide complex $K_2Ni(CN)_4$ increases prospects to engineer enzymes that are able to hydrolyze metallo-cyanides (Yanase et al. 2000). These studies will assist in forming a foundation for the use of CynD and CHT as templates for protein engineering and as candidates for use in detoxification of cyanide-containing industrial wastewaters.

Acknowledgements We gratefully acknowledge support from the Robert A. Welch Foundation, the University of Houston Environmental Institute, and the Gulf Coast Hazardous Substance Research Center, #069UHH0789, to M.J.B. and from the Wellcome Trust to B.T.S. for support of this project.

References

Basheer S, Kut ÖM, Prenosil JE, Bourne JR (1993) Development of an enzyme membrane reactor for treatment of cyanide-containing wastewaters from the food industry. Biotechnol Bioeng 41:465–473

Brenner C (2002) Catalysis in the nitrilase superfamily. Curr Opin Struct Biol 12:775–782

Brown DT, Turner PD, O'Reilly C (1995) Expression of the cyanide hydratase enzyme from *Fusarium lateritium* in *Escherichia coli* and identification of an essential cysteine residue. FEMS Microbiol Lett 134:143–146

Cluness MM, Turner PD, Clements E, Brown DT, O'Reilly C (1993) Purification and properties of cyanide hydratase from *Fusarium lateritium* and analysis of corresponding *chyI* gene. J Gen Microbiol 139:1807–1815

Dumestre A, Chone T, Portal JM, Gerard M, Berthelin J (1997) Cyanide degradation under alkaline conditions by a strain of *Fusarium solani* from contaminated soils. Appl Environ Microbiol 63:2729–2734

Fisher FB, Brown JS (1952) Colorimetric determination of cyanide in stack gas and waste water. Anal Chem 24:1440–1444

Fry WE, Munch DC (1975) Hydrogen cyanide detoxification by *Gloeocercospora sorghi*. Physiol Plant Pathol 7:23–33

Goldlust A, Bohak Z (1989) Induction, purification, and characterization of the nitrilase of *Fusarium oxysporum* f. sp. *melonis*. Biotechnol Appl Biochem 11:581–601

Harper DB (1977) Microbial metabolism of aromatic nitriles. Enzymology of C–N cleavage by *Nocardia* sp. (Rhodochrous group) N.C.I.B. 11216. Biochem J 165:309–319

Hook RH, Robinson WG (1964) Ricinine nitrilase II purification and properties. J Biol Chem 239:4263–4267

Ingvorsen K, Højer-Pedersen B, Godtrfedsen SE (1991) Novel cyanide-hydrolyzing enzyme from *Alcaligenes xylosoxidans* subsp *denitrificans*. Appl Environ Microbiol 57:1783–1789

Ingvorsen K, Godtfredsen SE, Højer-Pedersen B, Novo Nordisk (1992) Microbial cyanide converting enzymes, their production and use. United States Patent 5,116,744

Jandhyala DM, Berman M, Meyers PR, Sewell T, Willson RC, Benedik MJ (2003) CynD, the cyanide dihydratase from *Bacillus pumilus*: gene cloning and structural studies. Appl Environ Microbiol 69:4794–4805

Kobayashi M, Yanaka N, Nagasawa T, Yamada H (1992) Primary structure of an aliphatic nitrile-degrading enzyme, aliphatic nitrilase, from *Rhodococcus rhodochrous* K22 and expression of its gene and identification of its active site residue. Biochem 31: 9000–9007

Kobayashi M, Goda M, Sakayu S (1998) Nitrilase catalyzes amide hydrolysis as well as nitrile hydrolysis. Biochem Biophys Res Commun 253:662–666

Meyers PR, Rawlings DE, Woods DR, GG Lindsey (1993) Isolation and characterization of a cyanide dihydratase for *Bacillus pumilus* C1. J Bacteriol 175:6105–6112

Mudder T, Whitlock J (1984) Biological treatment of cyanidation wastewaters. Miner Metall Process 1:161–164

Nagasawa T, Mauger J, Yamada H (1990) A novel nitrilase, arylacetonitrilase, of *Alcaligenes faecalis* JM3. Purification and characterization. Eur J Biochem 194:765–772

Nakai T, Hasgawa T, Yamashita E, Yamamoto M, Kamasuka T, Ueki T, Nanba H, Ikenaka Y, Takahashi S, Sato M, Tsukihara T (2000) Crystal structure of *N*-carbamyl-D-amino acid amidohydrolase with a novel catalytic framework common to amidohydrolases. Structure 8:729–737

Nolan LM, Harnedy PA, Turner P, Hearne AB, O'Reilly C (2003) The cyanide hydratase enzyme of *Fusarium lateritium* also has nitrilase activity. FEMS Microbiol Lett 221:161–165

O'Reilly C, Turner PD (2003) The nitrilase family of CN hydrolyzing enzymes—a comparative study. J Appl Microbiol 95: 1161–1174

Richardson K, Clarke P, Imperial Chemical Industries (1993) Production of cyanide hydratase. United States Patent 5, 219, 750

Sewell BT, Meyers P, Berman M, Jandhyala DM, Benedik MJ (2003) The cyanide degrading nitrilase from *Pseudomonas stutzeri* AK61 is a twofold symmetric, 14-subunit spiral. Structure 11:1413–1422

Sexton AC, Howlett BJ (2000) Characterisation of a cyanide hydratase gene in the phytopathogenic fungus *Leptosphaeria maculans*. Mol Gen Genet 263:463–470

Stevenson DE, Feng R, Dumas F, Groleau D, Mihoc A, Storer AC (1992) Mechanistic and structural studies on *Rhodococcus* ATCC 39484 nitrilase. Biotechnol Appl Biochem 15:283–302

Wang P, VanEtten HD (1992) Cloning an properties of a cyanide hydratase gene from the phytopathogenic fungus *Gloeocercospora sorghi.* Biochem Biophys Res Commun 187:1048–1054

Wang P, Matthews DE, VanEtten HD (1992) Purification and characterization of cyanide hydratase from the phytopathogenic fungus *Gloeocercospora sorghi.* Arch Biochem Biophys 298: 569–575

Watanabe A, Yano K, Ikebukuro K, Karube I (1998a) Cyanide hydrolysis in a cyanide-degrading bacterium, *Pseudomonas stutzeri* AK61, by cyanidase. Microbiology 144:1677–1682

Watanabe A, Yano K, Ikebukuro K, Karube I (1998b) Cloning and expression of a gene encoding cyanidase from *Pseudomonas stutzeri* AK61. Appl Microbiol Biotechnol 50:93–97

Watanabe A, Yano K, Ikebukuro K, Karube I (1998c) Investigation of the potential active site of a cyanide dihydratase using site-directed mutagenesis. Biochim Biophys Acta 1382:1–4

Yanase H, Sakamoto A, Okamoto K, Kita K, Sato Y (2000) Degradation of the metal-cyano complex tetracyanonickelate (II) by *Fusarium oxysporum* N-10. Appl Microbiol Biotechnol 53:328–334