CYANIDE-DEGRADING ENZYMES FOR BIOREMEDIATION

A Thesis

by

LACY JAMEL BASILE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2008

Major Subject: Microbiology

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Approved by:

Chair of Committee,	Michael Benedik
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ABSTRACT

Cyanide-Degrading Enzymes for Bioremediation. (August 2008)

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Chair of Advisory Committee: Dr. Michael Benedik

Cyanide-containing waste is an increasingly prevalent problem in today's society. There are many applications that utilize cyanide, such as gold mining and electroplating, and these processes produce cyanide waste with varying conditions. Remediation of this waste is necessary to prevent contamination of soils and water. While there are a variety of processes being used, bioremediation is potentially a more cost effective alternative.

A variety of fungal species are known to degrade cyanide through the action of cyanide hydratases, a specialized subset of nitrilases which hydrolyze cyanide to formamide. Here I report on previously unknown and uncharacterized nitrilases from *Neurospora crassa, Gibberella zeae,* and *Aspergillus nidulans*. Recombinant forms of four cyanide hydratases from *N. crassa, A. nidulans, G. zeae,* and *Gloeocercospora sorghi* were prepared after their genes were cloned with N-terminal hexahistidine purification tags, expressed in *Escherichia coli* and purified using immobilized metal affinity chromatography. These enzymes were compared according to their relative specific activity, pH activity profiles, thermal stability, and ability to degrade cyanide in the presence of high concentrations of copper and silver.

Although all four were relatively similar, the *N. crassa* cyanide hydratase (CHT) has the greatest thermal stability and widest pH range where activity remained above 50%. *N. crassa* also demonstrated the highest rate of cyanide degradation in the presence of both metals tested. The CHT of *A. nidulans* and *N. crassa* have the highest reaction rate of the four fungal nitrilases evaluated in this work.

These data help determine optimization conditions for the possible use of these enzymes in the bioremediation of cyanide-containing waste. Similar to known plant pathogenic fungi, in vivo expression of CHT in both *N. crassa* and *A. nidulans* were induced by growth in the presence of KCN (potassium cyanide).

DEDICATION

To Alex, my sunshine on even the most cloudy days. To Dan, for being my biggest fan, even when I didn't deserve one. To Mom and Dad for a lifetime of love, encouragement, and support.

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CHAPTER I

INTRODUCTION

Cyanide

Cyanide is a nitrile, an organic compound that contains a triple-bonded carbon-nitrogen functional group. Most such compounds are highly toxic, carcinogenic, and mutagenic (4). Common symptoms of cyanide poisoning include gastric problems, vomiting, respiratory distress, convulsions, and coma (4). The toxicity of cyanide is quite high due to its ability to poison the respiratory system by inhibiting the final transport of electrons from cytochrome C oxidase to oxygen, preventing production of ATP.

Cyanide-containing waste is an increasingly prevalent problem in today's society. Cyanide comes from both natural and manufactured sources and occurs as both inorganic (HCN) and organic cyanide or nitriles (RCN) (33). An estimated 18 billion liters of cyanide containing waste are generated annually in the United States (7). With such applications as mining, electroplating, steel manufacturing, polymer synthesis, pharmaceutical production, and other specialized applications including dyes, and agricultural products (7), it is difficult to avoid its use. It is estimated that 834 thousand tons of hydrogen cyanide is required per year for use by these industries in the United States alone (7).

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This thesis follows the style of Applied and Environmental Microbiology.

Cyanide has been used for the extraction of gold from ore for almost a century (15). Cyanide forms tight complexes with heavy metals, such as gold, and dissolves them, allowing for the leaching of gold from ore (15). Cyanide and gold form a strong complex which allows for usage of relatively dilute sodium cyanide solutions (1, 15). The waste solutions created, however, are difficult to treat because of the cyanide forming complexes with other metals, such as iron (15). Iron, gold, and silver form very stable complexes that are resistant to treatment (7). Agriculture also contributes to the quantity of cyanide produced because of the use of nitrile pesticides such as dichlobenil and ioxynil which are pesticides used for rice, wheat, barley, corn, and berries (23). Bromoxynil and chlorothalonil are other examples of nitrile pesticides. These are used to control diseases of broad leaf crops (7). Chlorothalonil is used as an anti-fouling agent on the hulls of boats, but chlorothalonil rinses off of the hulls into the water, negatively impacting species other than the target fouling organisms (7).

Cyanide is produced naturally by many organisms. Plants are a significant source of cyanide compounds because of their use as a defense mechanism. When tissue injury occurs, cyanoglycosides are hydrolyzed to a sugar, HCN, and a keto or aldehyde compound (23). Arthropods, fungi, and bacteria can also produce cyanide (7). Fungi and bacteria use cyanide production to their advantage by secreting antimicrobial cyanide compounds which inhibit competing organisms (7). Insects, however, produce cyanide for use as a control over mating behavior (7). Although there are many organisms that produce cyanogenic compounds, these quantities do not compare to those produced by industrial processes.

Treatment Processes for Cyanide Waste

The majority of processes used for remediation convert cyanide into one or more less toxic compounds through an oxidation reaction (1). Sulfur dioxide/air is a common process developed by The International Nickel Company (INCO) more than two decades ago. This process uses SO_2 or a derivative along with air in the presence of a soluble copper catalyst. This causes oxidation of cyanide to the less toxic cyanate (1). The SO_2/air and the hydrogen peroxide processes, which are both catalyzed by copper, are the most successful of the non-biological processes (2).

The hydrogen peroxide treatment process is similar to the SO_2/air process, but hydrogen peroxide is used in place of SO_2 and air. The hydrogen peroxide process is primarily used for solutions, whereas the SO_2/air process can be used in both the treatment of slurry and solutions (1). A third process that is very important in the destruction of cyanide waste is alkaline and breakpoint chlorination. This process was at one point the most widely applied of all cyanide remediation processes. The first step of this two-step process is the conversion of cyanide to cyanogenic chloride. This compound is then hydrolyzed into cyanate (1).

There are also many biological alternatives for the treatment of cyanide containing waste. Although chemical and physical treatments provide more rapid detoxification and are less susceptible to environmental upsets, such as temperature, the decrease in expense and ease of use for biological alternatives are big advantages (2). Degradation of cyanide by microorganisms was first studied early in the twentieth century, but wasn't applied commercially until the mid-1980's at the Homestake Gold Mine (2). The

biological degradation process does not require addition of toxic or hazardous chemical, with the exception of small quantities of phosphoric acid required as a trace nutrient (2).

Nitrilases, such as cyanide hydratases and cyanide dihydratases, are examples of enzymes capable of cyanide degradation. There are numerous enzymes that are capable of degrading cyanide, but most have drawbacks that nitrilases do not have. Nitrogenases, found in nitrogen-fixing prokaryotes, require strictly anaerobic conditions because of oxygen's inhibitory effect on the enzyme (14, 16, 26). Rhodanese, an enzyme found in all animals, some plants, fungi, and prokaryotes, requires thiosulfate to function (52). This is because the enzyme catalyzes the transfer of a sulfur atom from a suitable donor, such as thiosulfate, to cyanide (10). This produces the less toxic thiocyanate. Nitrilases, however, have many properties that make them promising candidates for remediation. They are stable over long periods, require no co-factors, are readily expressed at high levels, and can function as purified enzyme, crude extracts, or within cells.

Nitrilase Superfamily

Nitrilases are part of the nitrilase superfamily and hydrolyze a variety of nonpeptide carbon-nitrogen bonds (34). Members of the nitrilase superfamily are found in all plants, animals, fungi, and some prokaryotes (34). There is sequence similarity among the members, but they have different substrate specificities and also have very diverse roles in biology, such as synthesis of signaling molecules, protein post-translational modification, and vitamin metabolism (8, 9). Brenner (2002) has classified the nitrilase

superfamily into 13 branches, with 9 having known or deduced specificity for certain nitrile- or amide-hydrolysis or amide-condensation reactions (34). Among all the branches, the proteins have a conserved catalytic triad of glutamic acid, lysine, and cysteine and all reactions involve the attack of a cyano or carbonyl carbon by this conserved cysteine (9).

The amidase reaction is seen most often among the branches with members of branches 2-4 performing this reaction, those in branches 5 and 6 being carbamylases, and branch 9 being N-acyltransferases, which perform the amidase reaction in reverse (34). Enzymes in branches 7 and 8 are also amidases (glutamine-dependent NAD synthetases), but are proposed to be specific for glutamine and branch 9 enzymes perform the amidase reaction in reverse (condensation) (34). Although branch 2-4 enzymes are all amidases, they all perform different functions. Branch 2 is a small group that consists of the aliphatic amidases which hydrolyze substrates such as the carboxyamide sidechains of glutamine and asparagine (34). The amino-terminal amidases of branch 3 also act on asparagine and glutamine, but these enzymes deaminate amino-terminal residues, producing aspartate and glutamate. This activity causes an increase in protein turnover rates (34). The biotinidases of branch 4 release biotin from such compounds as biotinamide (34). The carbamylase reaction carried out by enzymes of branches 5 and 6 is similar to the amidase reaction, but the end product of the carbamylase reaction is NH_2R instead of $RCOO^-$ due to the breakdown of pyrimidine bases and D-amino acids (34).

Enzymes of branches 10-13 have not been assigned substrate specificities. Branch 10 contains Nit, which was first identified as an extension on fly and worm homologs of the human Fhit tumor suppressor protein (34). Nit homologs are found in the same organisms that also contain Fhit homologs, but there are also Nit-related sequences in some prokaryotes that do not contain a Fhit homolog (34, 35). Although the structure of Nit has been determined, the substrate, along with other characteristics, is still unknown. By virtue of Rosetta stone relationships, which allow inference of the function of one protein by its fusion to a protein with a known function, it is assumed that Nit is involved in the function of Fhit (9, 34). The last three branches of the nitrilase superfamily have yet to be clearly defined. Branch 11 contains 13 putative enzymes of unknown specificity and branch 13 is a compilation of nonfused outliers (9). Branch 12 enzymes are proposed protein post-translational modifiers due to their fusion with N-terminal acetyltransferases (9).

Of the 13 branches that compose the nitrilase superfamily, only one (branch 1) includes enzymes that act on nitrile substrates (9). The enzymes classified into this branch are found in plants, animals, fungi, and many types of bacteria (34). The microbial nitrilases found in this branch convert nitriles, such as cyanide, to less harmful compounds through a hydration reaction. Cyanide hydratases (CHT), found in numerous plant pathogenic fungi such as *Fusarium solani* (6)*, Gloeocercospora sorghi* (48), *Fusarium lateritium* (11, 32)*,* and *Leptosphaeria maculans* (43), convert cyanide to formamide (11). The related cyanide dihydratases (CynD) convert cyanide to formate and ammonia (50) and are found in *Alcaligenes xylosoxidans* subsp. *dentrificans* (18)*,*

Bacillus pumilus (28)*,* and *Pseudomonas stutzeri* AK61 (51). As shown in Figure 1 below, cyanide dihydratases can catalyze both hydrolysis steps, but cyanide hydratases require the organism producing the hydratase to also produce an amidase for the subsequent hydrolysis to ammonia.
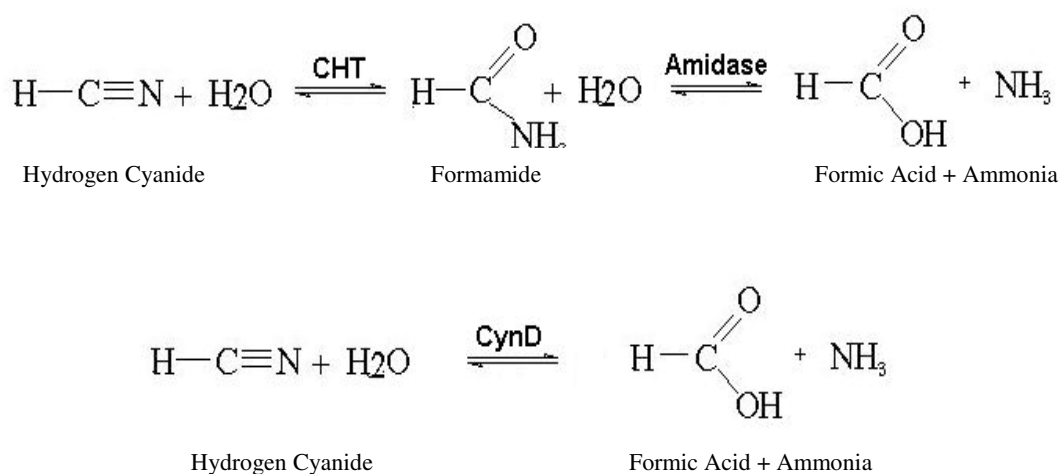
Figure 1: Mechanism of cyanide hydrolysis by cyanide hydratase (top) and cyanide dihydratase (bottom). Cyanide hydratase produces formamide, requiring an amidase for the subsequent hydrolysis to formic acid. Cyanide dihydratase hydrolyses cyanide to formic acid.

Nitrilases

The first nitrilase, which was isolated from barley leaves, was described by Thimann and Mahadevan in 1964 and catalyzed the conversion of indoleacetonitrile to indoleacetic acid (33, 45). The first bacterial nitrilase isolated was likely from a *Pseudomonas* species that was found in a soil sample by selection for grown on ricinine, a naturally occurring nitrile (33). Nitrilases in general show activity with a broad range of nitrile substrates, however, CHT and CynD show high specificity for hydrogen

cyanide, but show very little activity with nitriles. (33). Although they are known to hydrolyze nitriles to their corresponding acid and ammonia, the natural substrates of most nitrilases have not been identified (39). Nitrilases also show notable differences when compared based on substrate specificity, native structure, and pH optima (33).

Nitrilases have been classified into three categories based on substrate specificity (24). Aromatic nitrilases degrade aromatic nitriles and heterocyclic nitriles isolated from plants. Organisms such as *Nocardia* sp. and *Fusarium solani* have been shown to degrade aromatic nitriles, such as benzonitrile (24). Although aromatic nitriles can be degraded in one step by nitrilase, aliphatic nitrilases are degraded in two stages. First is the conversion to the corresponding amide, followed by conversion to the acid plus ammonia (24). Nitrilases that degrade aromatic nitriles are unable to act on aliphatic nitriles (24). It has been found that *Rhodococcus rhodochrous* K22 shows broad substrate specificity toward unsaturated aliphatic nitriles, such as acrylonitrile (24). Finally, there is a new type of microbial nitrilase found in *Alcaligenes faecalis* JM3 that is classified as an arylacetonitrilase (24). *A. faecalis* JM3 can act on acetonitrile and acrylonitrile, which are aromatic and aliphatic nitriles, respectively. These are the only non-arylacetonitriles that this enzyme can degrade. Arylacetonitriles include indole-3-acetonitrile, phenylacetonitrile, and thiopheneacetontrile (24).

Nitrilases, such as those from *A. faecalis* ATCC 8750 and *Acinetobacter* sp., are also important industrial enzymes because of their ability to produce biologically active enantiomers, such as S-(+)-1-(4'-isobutylphenyl) propionic acid (S-(+)-ibuprofen) and R-(-) mandelic acid (4, 24, 33). Arylacetic acids, which are important pharmaceutical

intermediates, can be produced in enantiomerically pure form by the hydrolysis of their corresponding nitrile (3).

Another application of nitrilases is in transgenic crops. The nitrilase from *Klebsiella ozaenae* is active on the herbicide bromoxynil (24). This aromatic nitrilase inactivates the herbicide by hydrolyzing its cyano group. Expression of this nitrilase gene in plants provides tolerance to this herbicide without hindering growth (24).

Nitrile hydratases (NHases) hydrolyze cyanide and other nitriles using a mechanism similar to that of cyanide hydratases. Both nitrilases and nitrile hydratases are important as potential catalysts for production of amides and acids from their corresponding nitriles, but NHases are the most widely applied of the two (23). *Rhodococcus rhodochrous* J1 has been used in the production of acrylamide and nicotinamide and is the first successful example of bioconversion for the manufacture of a commodity chemical (23). Since beginning production in the early 1990's, the industrial production of acrylamide from acryonitrile has reach 30,000 tons per year (23). The advantage of bioconversion over chemical processes is that pH and temperature conditions are much less severe and very pure products are produced without the presence of secondary by-products (24).

Quaternary Structure

The native size for these enzymes has been determined by multiple methods and the results suggest that several microbial nitrilases are high molecular weight homo-oligomers with subunit numbers ranging from 10 to 18 (41). Active CHT from *G.*

sorghi is believed to be greater than 300 kDa, with the monomers being approximately 40 kDa (49). The enzymes from *F. lateritium* and *F. solani* have been shown to have similar molecular masses, but the enzyme from *L. maculans* is 160 kDa (33).

Another characteristic of microbial nitrilases is their unusual quaternary structure. Native proteins have sizes in excess of 500 kD, with subunits ranging from 35-40 kD. They are homomultimers arranged in a helical shape and are generally 10, 14, or 18 subunits or nonterminating. Oligomer formation has been suggested to be necessary for activity and two lines of evidence support this conclusion. The nitrilases in Rhodococci are found as inactive dimers. It has been shown that these dimers form active decamers in the presence of their substrate, benzonitrile (30). Also, the cyanide dihydratase of *B. pumilus* has been demonstrated to switch from an 18mer to form long helical fibers at pH 5.4. This fiber formation correlates with a small increase in activity, consistent with a model whereby the terminal monomers of the short spirals become activated as they join in the extended fibers (42). Jandhyala et al. (2005) speculates that this can be explained by inactive terminal subunits. As more long spirals form, this causes less inactive terminal subunits in the overall population (21).

The atomic structure has been solved for four unrelated nitrilases (42). These differ from microbial nitrilases in that they do not form typical large homo-oligomeric complexes. The hypothetical protein PH0642 from *Pyrococcus horikoshii* and the putative CN hydrolase P018 from yeast both exist as dimers (25, 40); however, N-carbamyl-D-amino acid amidohydrolase (DCase) and the Nit domain of Nitfhit are both tetrameric (31, 35). The substrate of Nit (branch 10) is unknown, but DCase (branch 6)

is a carbamylase. Neither of these enzymes degrades cyanide, but their structures were used to produce a partial atomic model that could be fitted to the CynD of *P. stutzeri* (41). This was done using the Nit tetramer, which has two possible dimer surfaces perpendicular to each other (35, 41). Based on the most probable dimer surface of these two, a model was proposed for the *P. stutzeri* cyanidase. This dimeric model was used to fill the density that was determined using negative stain electron microscopy, suggesting the enzyme to be a 14-subunit spiral (Figure 2A). Figure 2B represents the three-dimensional reconstruction of negatively stained micrographs of the *P. stutzeri* cyanide dihydratase. Similar three-dimensional reconstructions of *Bacillus pumilus* suggest it is an 18-subunit spiral and is comprised of dimers with each subunit being 37kDa (20). The monomer unit of most nitrilases has a molecular mass of 32-45 kDa (33).

Summary

Microbial nitrilases are the most promising candidates for remediation; however, the many uses of cyanide produce a wide variety of waste products with varying traits, such as pH and concentration of other contaminants. All of these factors must be taken into consideration when trying to determine the optimal enzyme for the remediation of cyanide-containing waste.

The three known bacterial cyanide dihydratases, those of *Alcaligenes xylosoxidans* subsp. *dentrificans* DF3, *Bacillus pumilus* C1, and *Pseudomonas stutzeri* AK61, have been studied for use in industrial cyanide remediation. Along with these, multiple fungal

cyanide hydratases have also been studied. The fungal nitrilases that have been characterized to date include *Fusarium lateritium* (11)*, Gloeocercospora sorghi* (48, 49)*, Leptosphaeria maculans* (21)*,* and *Fusarium solani* (5)*. F. lateritium* and *A. xylosoxidans* subsp. *dentrificans* have been patented for use as granular biocatalysts for the detoxification of cyanide-containing waste (17, 38).
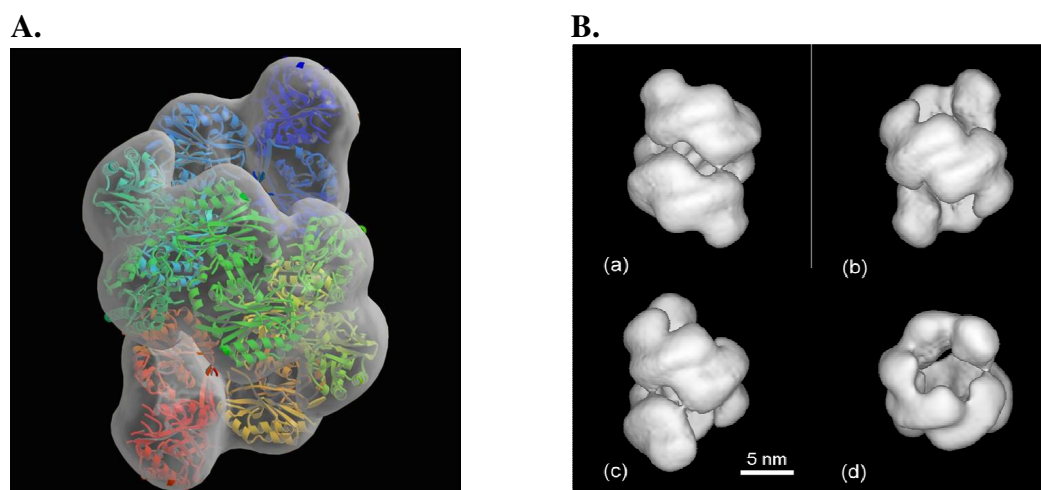

Figure 2: Model of *Pseudomonas stutzeri* AK61 nitrilase. This was determined by fitting 14 subunits to the density determined by negative stain electron microscopy (A) and 3D reconstruction of negative stain electron micrographs (B). Taken from Sewell et al. 2003.

Genome searching revealed that a variety of non-plant pathogenic fungi, including human pathogens such as *Aspergillus fumigatus,* carry related genes. These genes from lab strains of the saprophytic fungi *Aspergillus nidulans* and *Neurospora crassa* as well as a plant pathogenic fungi *Gibberella zeae* were cloned and compared them to the previously characterized nitrilase from *G. sorghi* (21, 48, 49).

The many uses of cyanide produce a wide variety of waste products with varying qualities, such as pH and concentration of other contaminants. This work compares these four cyanide hydratases with respect to their relative stabilities, rate of cyanide degradation, and ability to work under pH conditions expected for cyanide containing waste.

CHAPTER II

CLONING AND PURIFICATION OF HETEROLOGOUS NITRILASES

Overview

Described in this chapter are the methods used to clone, express, and purify the cyanide hydratase of *N. crassa*, *A. nidulans*, and *G. zeae*. The protein sequence of the CHT from *G. sorghi,* which has previously been characterized (48), was used to perform a BLAST search. From the results, *N. crassa* and *A. nidulans* were chosen because they are non-pathogenic fungi, unlike the other organisms with a CHT that has previously been characterized. *G. zeae* (*Fusarium graminearum*) was interesting because its CHT had not been characterized, but is similar to other *Fusarium* species which have been, enabling comparison among closely related cyanide hydratases.

Materials and Methods

Bacterial Strains and Plasmids

The plasmids and strains used are described in Table 1. The *N. crassa* knockouts were provided by the Fungal Genetics Stock Center as part of their comprehensive gene knockout project (27).

Table 1 Strains and Plasmids

Strain/Plasmids	Description/Genotype	Reference
E. coli		
MB1837	BL21(DE3), pLysS F⁻ *ompT hsdS*$_B$ *gal dcm*(DE3)	Novagen
MB3436	MM294 but *lacI*^{q} *lacZ* ΔM15	Lab stock
MB3487	MB1837 p3487	This work
MB3643	MB1837 p3639	This work
MB3644	MB1837 p3641	This work
MB3653	MB1837 p3649	This work
Plasmids		
pET-26b	Kan^R *E.coli* expression vector (C-terminal His-tag)	Novagen
pET-28a	Kan^R *E.coli* expression vector (N-terminal His-tag)	Novagen
pBluescript II KS+	Amp^R *E.coli* cloning vector	Stratagene
pBC SK+	Cam^R cloning vector	Stratagene
p2160	pBS KS+ with *NdeI* replacing the *ClaI*	Lab stock
p2313	pET26b *NdeI-XhoI* fragment of *G. sorghi cht*	Jandhyala et al. 2005
p3460	pET26b *NdeI-EcoRI* fragment of *N. crassa cht*	This work
p3487	pET28a *NdeI-EcoRI* fragment of *N. crassa cht*	This work
p3622	pET26b *NdeI-HindIII* fragment of *G. zeae cht*	This work
p3639	pET28a *NdeI-XhoI* fragment of *G. sorghi cht*	This work
p3641	pET28a *NdeI-HindIII* fragment of *G. zeae cht*	This work
p3646	pET26b *NdeI-EcoRI* fragment of *A. nidulans cht*	This work
p3649	pET28a *NdeI-EcoRI* fragment of *A. nidulans cht*	This work
Neurospora crassa		
FGSC#2489	Mating Type A *N. crassa* 74-OR23-1VA	McCluskey 2003
FGSC#9718	Mating Type a delta mus-51::bar+	McCluskey 2003
FGSC#11824	Locus Tag NCU04697.2	McCluskey 2003
FGSC#11825	Locus Tag NCU04697.2	McCluskey 2003
Aspergillus nidulans		
FGSC #A4	Glasgow wild type (veA+)	FGSC

Culture Media and Reagents

Escherichia coli strains were grown in LB broth containing 0.05% glucose, 0.5% glycerol (0.2% lactose added to induce protein expression) which is essentially the autoinduction media of Studier (44). Antibiotics were added to concentrations of 100 μg mL^{-1} ampicillin, 25 μg mL^{-1} chloramphenicol, and 25 μg mL^{-1} kanamycin, for selection

in *E. coli* strains. *N. crassa* strains were grown in 1X Vogel's medium (47). *A. nidulans* was grown in Complete Medium (22).

DNA Manipulations

The cyanide hydratase gene was amplified from *A. nidulans* and *G. zeae* genomic DNA and a cDNA library of *N. crassa* using PfuTurbo® Hotstart PCR Master Mix (Stratagene, La Jolla, CA). The primers used introduced an *NdeI* site at the ATG codon and a unique site in the downstream primer (beyond the stop codon) for cloning into p2160. These clones were sequenced and later moved by subcloning into pET26b to produce untagged protein and pET28a to generate N-terminal His-tagged fusions.

Removal of introns was necessary for the genomic *A. nidulans* and *G. zeae* clones. The *A. nidulans* gene contains three introns and *G. zeae* contains two. The QuikChange® Site-Directed Mutatgenesis Kit (Stratagene, La Jolla, CA) was used to create deletions of the intron segments, producing the predicted proteins shown in Figure 3. All constructs were introduced into MB3436 for routine cloning and the *E. coli* B strain BL21(DE3)pLysS (MB1837) was used for expression from the T7 promoter.

Expression and Purification of CHT from *E. coli*

Protein production from strains MB3487, MB3643, MB3644, and MB3653 was achieved in 50mL cultures of autoinduction medium with lactose containing $25\mu g\ mL^{-1}$ kanamycin and grown overnight at $30^{o}C$.

After centrifugation, cell pellets were resuspended in 20 mL of 20mM sodium phosphate (pH 7.4), 100mM NaCl, 12.5mM imidazole (pH 7.4), and $1mg\ mL^{-1}$

lysozyme. After incubation on ice for 15 minutes, cells were lysed by five cycles of freezing at -80 ^{o}C, and thawing. Viscous DNA was removed by the addition of a crude preparation of *Serratia* nuclease (29) before centrifugation at 3750 rpm for 15 minutes. The supernatant was then clarified with a 0.45μm filter.

The hexahistidine-tagged cyanide hydratase enzymes were purified from crude cell lysates by immobilized metal affinity chromatography using a 1mL precharged HisTrap™ Ni Sepharaose™ High Performance column (Amersham Biosciences, Piscataway, NJ). The column was washed with 5 column volumes of water and 10 column volumes of buffer B (25mM imidazole, 1mM NaCl, and 20mM sodium phosphate) before passing the filtered lysate through the column. The column was then washed with 10 column volumes of buffer B with 100mM imidazole. Enzyme was eluted using 10 column volumes of buffer B with 500mM imidazole. Peak fractions having cyanide-degrading activity were pooled and stored at 4°C.

Results

Cloning for Expression in *E. coli*

The *cht* gene of *N. crassa, A. nidulans,* and *G. zeae* were PCR-amplified from a cDNA library of *N. crassa* or the genomic DNA of *A. nidulans* and *G. zeae*, respectively. The resulting products were then subcloned into p2160 and transformed into MB3436 to allow for blue-white screening. Introns were deleted from the *A. nidulans* and *G. zeae* clones by site-directed mutagenesis using the Quick-change protocol. When the introns from the *A. nidulans* gene were deleted according to the

NCBI entry (AN8773.2) nitrilase open reading frame, it did not have activity. Upon comparison with other nitrilases it was obvious the intron assignment was likely not correct. A more likely sequence was then deduced from this comparison, especially the with the closely related *A. fumigatus*. This new ORF, that shown in Figure 3, was active against cyanide. The DNA of each final clone was verified. The cDNA clone for *G. sorghi* was already in our collection (21).

All four were then subcloned using the *NdeI* site of pET28a to create N-terminal His-tagged constructs and likewise in pET26b to generated untagged proteins. The final confirmed constructs were transformed into BL21 (DE3) pLysS. All strains expressed cyanide-degrading activity shown by measuring loss of cyanide. High level expression of nitrilase was easily visualized using SDS-PAGE. Expression levels and enzyme activity was comparable from both the His-tagged and untagged versions of these enzymes.

<u>Purification of CHT Enzymes</u>

CHT was purified from MB3487, MB3643, MB3644, and MB3653 using immobilized metal affinity chromatography as described in Methods, and SDS-PAGE was used to analyze the purity and relative concentration of the protein preparations. After staining, the nitrilase was the dominant band and there were very few background bands visible (data not shown). Protein preparations were stored at 4°C because storage at -80°C resulted in precipitation and loss of activity. The enzymes were stable for many months at 4°C.

```
N.crassa    --MVLTKYKAAAVTSEPCWFDLEGGVRKTIDFINEAGQAGCKLVAFPEVWIPGYPYWMWK 58
G.zeae      --MAITKYKAAAVTSEPGWFDLEGGVRKTIDFINEAGQAGCKFVAFPEVWIPGYPYWMWK 58
G.sorghi    --MPINKYKAAVVTSEPVWENLEGGVVKTIEFINEAGKAGCKLIAFPEVWIPGYPYWMWK 58
A.nidulans  MSPVLKKYKAAAVNAEPGWFDLEESVRRTIHWINEAGRNRCKLIAFPELWIPGYPYWMWK 60
              :.*****.*.:** * :** .* :**.:*****:  **::****:***********

N.crassa    VTYQQSLPMLKKYRENAMAVDSDEFRRIRRAARDNQIYVSLGFAEIDHATLYLAQALIDP 118
G.zeae      VTYLQSLPMLKRYRENSMAVDSEEMRRIRRAARDNQIFVSLGFSEIDHATLYLSQVLIGP 118
G.sorghi    VNYLQSLPMLKAYRENSIAMDSSEMRRIRAAARDNQIYVSIGVSEIDHATLYLTQVLISP 118
A.nidulans  VNYQESLPLLKKYRENSLLSDSEEMRRIREAARANKIYVSLGYSEVDLASLYTTQVLISP 120
            *.* :***:** ****::  **.*:**** *** *:*:**:* :*:* *:** :*.**.*

N.crassa    TGEVINHRRKIKPTHVEKLVYGDGAGDTFMSVTPTELGRLGQLNCWENMNPFLKSLNVSM 178
G.zeae      DGAVINHRRKIKPTHVEKLVYGDGAGDTFMSVSETEIGRVGQLNCWENMNPFLKSLNVSA 178
G.sorghi    LGDVINHRRKIKPTHVEKLVYGDGSGDSFEPVTQTEIGRLGQLNCWENMNPFLKSLAVAR 178
A.nidulans  AGNILNHRRKIRATHVERLVFGDGTGDTTESVVQTEIGRVGHLNCWENMNPFMKSYAASL 180
             * ::******:.****:**:***:**:  .*  **:**:*:************:** .:

N.crassa    GEQIHIAAWPIYPGKETLKYPDPATNVADPASDLVTPAYAIETGTWTLAPFQRLSVEGLK 238
G.zeae      GEQVHVAAWPVYPGKERQVYPDPATNYADPASDLVTPEYAIETGTWTLAPFQRLSVEGLK 238
G.sorghi    GEQIHVAAWPVYPDLSKQVHPDPATNYADPASDLVTPAYAIETGTWVLAPFQRISVEGLK 238
A.nidulans  GEQVHVAAWPLYPGKETLKYPDPYTNVAEANCDLVTPAYAIETGTYTLAPWQTITEEGIK 240
            ***:*:****:**. .   :*** ** *:. .***** *******:.***:* ::  **:*

N.crassa    KNTPEGVEPETDPSTYNGHARIYRPDG-SLVVRPDKDFDGLLFVDIDLNECHLTKALADF 297
G.zeae      INTPEGVEPETDPSVYNGHARIYRPDG-SLVVKPEKDFDGLLFVDIDLNECHLTKVLADF 297
G.sorghi    RHTPPGVEPETDATPYNGHARIFRPDG-SLYAKPAVDFDGLMYVDIDLNESHLTKALADF 297
A.nidulans  LNTPPG-KPLEDPNIYNGHGRIFAPDGRNLVPHPAKDFQGLLYVDIDLDEIHLTKSLADF 299
             :** * :*  *.. ****.**: *** .*  :*  **:**::*****:* **** ****

N.crassa    AGHYMRPDLIRLLVDTSRKELVTEVD--RNGGIVQYSTRERLGLNTPLEND--KEGKK-- 351
G.zeae      AGHYMRPDLIRLLVDTRRKELITEAD--PNGSIATYSTRQRLGLDKPLEK---KEGEDTP 352
G.sorghi    AGHYMRPDLIRLLVDTRRKELVTEVGGGDNGGIQSYSTMARLGLDRPLEEEDYRQGTDAG 357
A.nidulans  GGHYMRPDLIRLLVDGNRKDLVVSEDR-INGGIKYTSTMDRVGLTKPLEAP---KPTDQK 355
            .*************  **:*:.. .   **.*    **  *:** ***     :  .

N.crassa    -----------
G.zeae      DVL-------- 355
G.sorghi    ETEKASSNGHA 368
A.nidulans  E---------- 356
```

Figure 3: Alignment of our sequences of the four fungal cyanide-degrading nitrilases using ClustalW. Identical residues noted by *, similar amino acids by **:** and less similar by a dot. Non-conservative charges are left blank below the alignment. The catalytic triad residues are highlighted in blue.

CHAPTER III

BIOCHEMICAL CHARACTERIZATIONS

Overview

The three enzymes discussed in the previous chapter were characterized along with the previously cloned CHT of *G. sorghi*. This chapter discusses the methods and comparison of results based on pH profiles, thermal stability, enzyme parameters, and the ability to degrade cyanide in the presence of high concentrations of either copper or silver. *N. crassa* and *A. nidulans* strains were also tested to determine if production of CHT is induced by growth in the presence of KCN (potassium cyanide).

Materials and Methods

<u>pH Activity Profiles</u>

The pH profiles for the purified cyanide hydratases of G. *sorghi, G. zeae, N. crassa*, and *A. nidulans* were determined using sodium phosphate buffers between pH 4.5 to pH 11. A 1M stock solution of KCN was diluted in 100 mM MOPS, pH 7.6 to yield a final KCN concentration of 100 mM. Reactions were run in duplicate at room temperature in a final volume of 300μL consisting of 100 mM buffer, enzyme diluted in the same pH buffer, and 10mM KCN. Enzyme concentrations used for rate measurements were: 1.8ug mL^{-1} for *N. crassa*, 3.5ug mL^{-1} for *G. sorghi* (10-fold more at pH 11), 2.2ug mL^{-1} for *A. nidulans* (10-fold more at pH 10.5 and 11), and 6.9ug mL^{-1} for *G. zeae* (10-fold more at pH 4.5 and 11). Reactions were run for up to 61 minutes with

the baseline sample taken at 1 minute and at subsequent time points where a measurable amount of cyanide still remained. The picric acid endpoint method for determining cyanide concentration was used to determine the rate of degradation (13). To each 0.1ml sample 0.2mL of 0.5% picric acid in 0.25M sodium carbonate was added and heated to $100^{o}C$ for 6 minutes. The undiluted sample (3µL) was used to measure the absorbance at 520nm on a NanoDrop spectrophotometer and determine the change in OD per minute between the 1 min baseline and the subsequent time point. Controls containing cyanide without enzyme were run at each pH.

Determination of Thermal Stability

To determine their thermal stability, each enzyme was incubated at temperatures of 27, 37, 43, and $50^{o}C$ and samples were taken at time points ranging from 30 minutes to 48 hours. The activity of each enzyme sample was then measured at room temperature in 0.1M MOPS, pH 7.4 using 10mM KCN. All assays were performed in duplicate. Enzyme concentrations in the assay initially were the same as for pH profiles, but were increased as needed due to loss of activity upon incubation at the higher temperatures. Assays were performed as described for the pH profiles.

Enzyme Parameters

Enzyme was diluted in 1mL 0.1M MOPS, pH 7.6 and KCN from a 1M, pH 7.6 stock, was added to final concentrations of 20, 40, 60, 80, 100, 120, and 140mM. Upon the addition of cyanide, each reaction was divided into 3 tubes of 300µL each. Reactions were run for 61 minutes at room temperature with samples being taken at 1, 6, 11, 31

and 61 minutes. Enzyme concentrations used for rate analysis were 0.61ug mL^{-1} for *N. crassa*, 1.36ug mL^{-1} for *G. sorghi*, 0.98ug mL^{-1} for *A. nidulans*, and 1.20ug mL^{-1} for *G. zeae*. The picric acid assay loses linearity above ~12mM; therefore all cyanide concentrations above 10mM were diluted 10-fold before adding picric acid except 120 and 140 mM were diluted 20-fold. After boiling for 6 minutes, 700µL of water was added to the reaction. The reactions were diluted in half into 1mL cuvettes and the OD520 was measured. The change in OD was calculated using the same method as with the pH profiles.

Cyanide Induction of Fungal Cultures

Overnight *A. nidulans* cultures were prepared in 2mL of complete medium (22) inoculated 1:100 with *A. nidulans* spores. After growing overnight, the culture was diluted 5-fold to a final volume of 1mL in two separate tubes. To one tube, KCN was added to 0.1mM for induction. The cultures were grown overnight, then harvested, washed, and sonicated.

N. crassa cultures of the wild type (FGSC #2489 and FGSC #9718) and *cht* knockout strains (FGSC #11824 and FGSC #11825) of both mating types, provided by the Fungal Genetics Stock Center, were similarly grown overnight at 30°C in 1X Vogel's + 1.5% glucose. The cultures were harvested, washed, and each resuspended in two tubes 1X Vogel's + 1.5% glucose and two tubes 1X Vogel's + 1.5% glucose without any nitrogen source. 2mM KCN was added to one tube of each set. After growing for 3 hours at 30°C, the cultures were washed and resuspended in 4mL 0.1M MOPS and then sonicated. Both *A. nidulans* and *N. crassa* were assayed with 5mM KCN.

Measurement of Copper and Silver Effects

Each enzyme was assayed for cyanide degradation in waste water from silver or copper electroplating baths taken from an electroplating plant in Houston, Texas. These samples contain high amounts of either copper or silver and have cyanide concentrations of approximately 1M and pH in excess of 11. Each water sample was diluted 10-fold to approximately 100mM cyanide in 1M MOPS, pH 7.6. This also lowered the pH to ~8 so that pH would have no influence on activity. For use as a control, laboratory stocks of KCN were also diluted to 100mM in 1M MOPS, pH 7.6. For use as a measurement of cyanide concentration at time point zero, all three diluted cyanide samples (KCN, CN + copper, and CN + silver) were set up with no enzyme present. For each cyanide sample, enzyme was added and incubated at room temperature for 24 hours. Total volume for each reaction was 100μL. Enzyme concentrations used were 7.19ug mL^{-1} for *N. crassa*, 7.01ug mL^{-1} for *G. sorghi*, 8.86ug mL^{-1} for *A. nidulans*, and 13.71ug mL^{-1} for *G. zeae* for the KCN and silver assays. Ten-fold more enzyme was used for the copper assays. After 24 hours, the picric acid endpoint method was used and OD$_{520}$ measured to determine the percent cyanide degraded.

Results

pH Activity Profile of CHT Enzymes

The pH activity profiles of purified *G. sorghi, G. zeae, N. crassa*, and *A. nidulans* CHT were measured at pH's ranging from 4.5 to 11 (Figure 4). All of these displayed maximum activity between pH 6-7, with an abrupt decrease in activity above pH 10 and

below their individual maxima. CHT from *G. sorghi* and *G. zeae* displayed greater than

50% of activity relative to their maximal throughout the range of pH 6-8.5, the narrowest

range of activity. *N. crassa* CHT had greater than 50% maximal activity over the widest

range, which was between pH 5 and 9. *A. nidulans* CHT also had a relatively wide range

of activity, but at all points, the activity was generally lower than *N. crassa* except at pH

values below 5.5.

Thermal Stability

    To determine the stability of each enzyme, they were incubated at 27, 37, 43, and

50°C and the activity measured at time points ranging between 0 and 48 hours (Figure

5). All four enzymes retained greater than 70% of their relative activity after 48 hours at

27°C and dropped below 5% after 48 hours at 50°C. When comparing activity at 37°C

and 43°C, CHT from *N. crassa* was most stable with no loss of activity after 48 hours at

37°C, and about 40% of activity remaining after 48 hours at 43°C. *G. zeae* CHT showed

an initial increase in activity at lower temperatures, but the subsequent stability was

similar to *N. crassa* CHT. Both of these enzymes showed a gradual decrease in activity

at 50°C. The CHT of *A. nidulans* and *G. sorghi* quickly lost activity at the two highest

temperatures and 50% of activity was lost after 48 hours at 37°C.
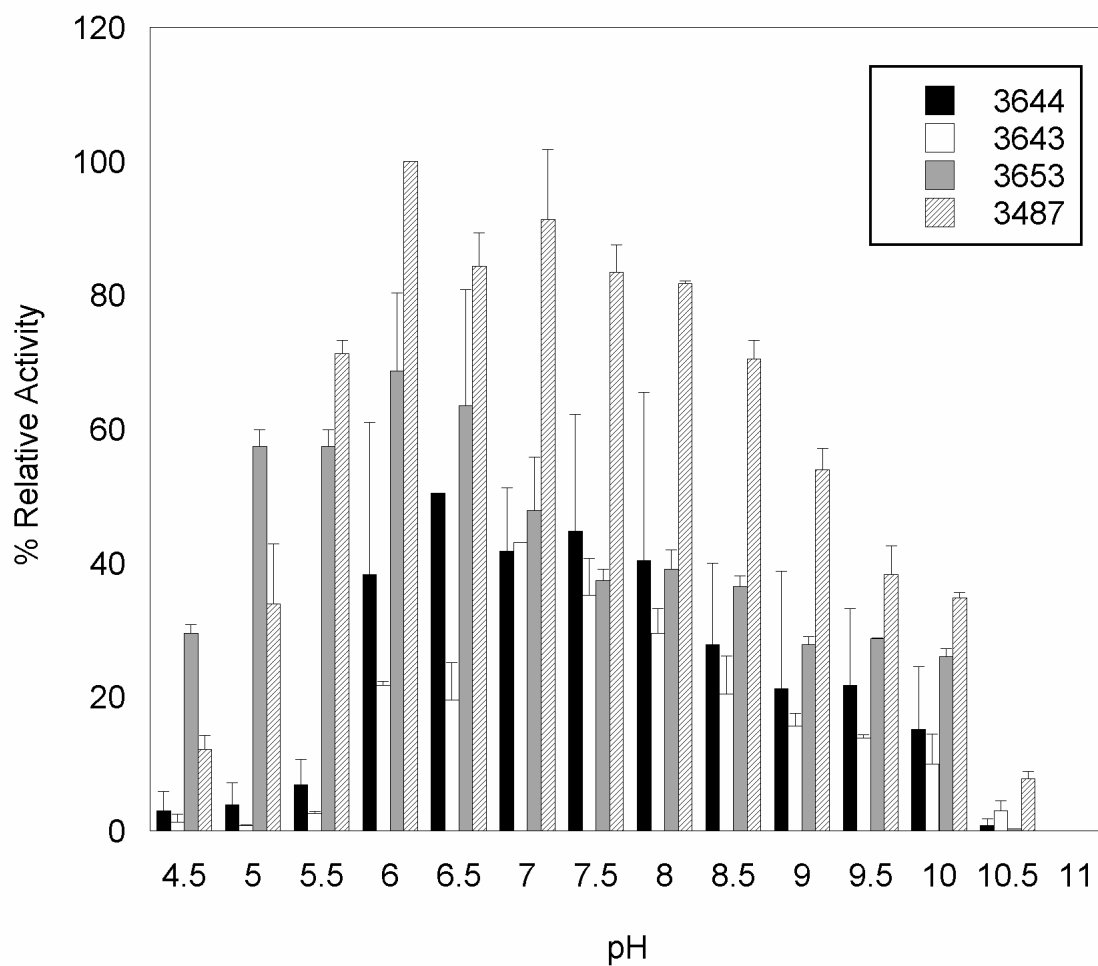
Figure 4: pH activity profiles of CHT from *N. crassa* (MB3487), *A. nidulans* (MB3653), *G. zeae* (MB3644), and *G. sorghi* (MB3643). Error bars represent half the value range from duplicate experiments and the columns represent the average.
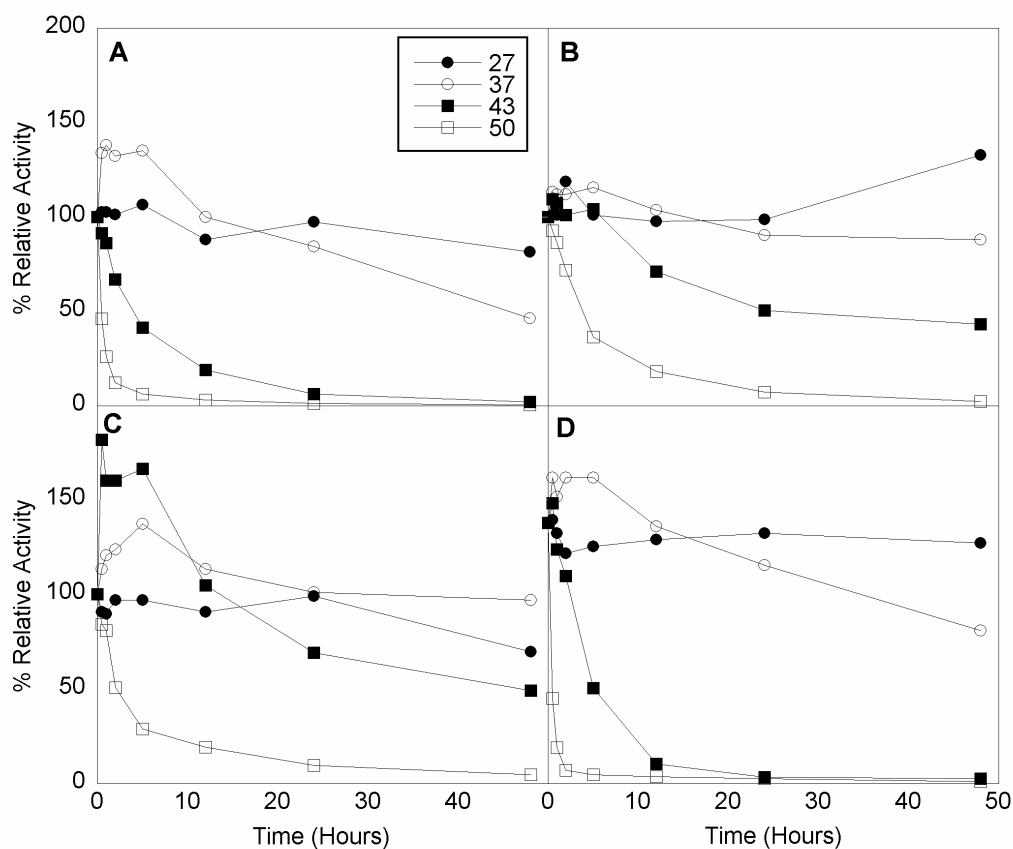
Figure 5: Thermal stabilities of CHT from a) *G. sorghi*, b) *N. crassa*, c) *G. zeae,* and d) *A. nidulans.* Shown is the activity remaining after incubation at the noted temperatures for periods between 0 and 48 hours. Each point is calculated as the percentage of the 0 time point which is set as 100%. Each point represents that average from duplicate experiments.

Enzyme Parameters

Originally, I set out to compare the kinetic parameters of each enzyme. After plotting the data, I observed that the rate of the reaction decreased at high substrate concentrations in the case of all the enzymes (Figure 6). This precluded a determination of K_m and V_{max} values for the recombinant CHT enzymes from classic Michaelis-Menten kinetics. The graphs of *N. crassa* and *A. nidulans,* show that the enzyme reaches

a maximum and then the rate drops with further increase in substrate concentration. These results are suggestive of substrate inhibition, however, the curves fail to fit a classic substrate inhibition model (T. Sewell, personal communication).
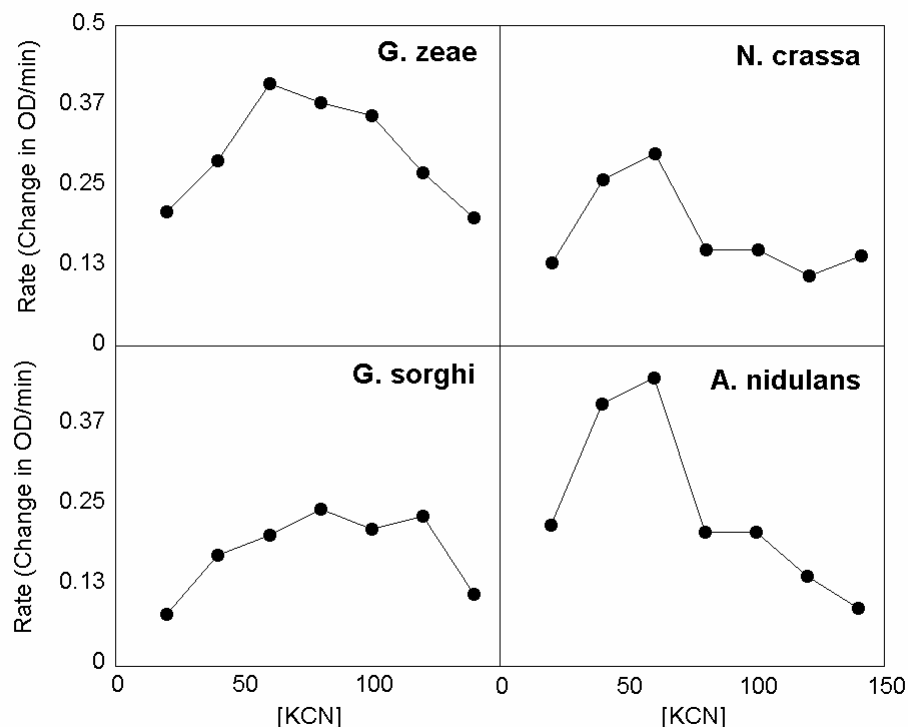
Figure 6: Enzyme inhibition at high substrate concentrations. Michaelis-Menten plots of rate vs. substrate concentrations of the four cyanide hydratases. The points are averages of triplicate experiments.

The data were used to determine reaction rates of the four enzymes which are listed in Table 2. At a single substrate concentration, 20mM KCN, the *A. nidulans, N. crassa,* and *G. zeae* CHT have the highest rates at 0.22, 0.21, and 0.18 ΔOD/min/μg enzyme, respectively. The *G. sorghi* CHT is the lowest of the group by over 3-fold.

With the exception of *G. sorghi*, all of the enzymes reach their maximum rate at 60mM

KCN and have relatively similar maximum rates, with *N. crassa* having the highest rate.

G. sorghi does not reach maximum activity until it reaches 80mM KCN and has a much

lower maximum rate than the other three enzymes.

Table 2. Reaction Rates

Enzyme	Rate at 20mM[a]	Maximal Rate[b]	[KCN][c]
N. crassa	0.21 ± 0.02	0.49 ± 0.05	[60]
A. nidulans	0.22 ± 0.04	0.46 ± 0.09	[60]
G. zeae	0.18 ± 0.01	0.34 ± 0.04	[60]
G. sorghi	0.06 ± 0.03	0.18 ± 0.06	[80]

Rates are ΔA_{540}/min/µg enzyme. The rate for all enzymes at a single substrate concentration (a). The maximal rate (b) at the substrate concentration (c) is shown.

Regulation of Cyanide Hydratase Expression

Cultures of *N. crassa* wild type strains of both mating types (FGSC #2489) as

well as a knockout mutant at the *cht* gene locus generated by the Fungal Genetics Stock

Center (FGSC #11825) were grown in minimal media as described. The effect of

cyanide was tested by adding 2mM KCN to one set of cultures. The effect of exogenous

ammonia was also tested for *N. crassa* because the breakdown of cyanide leads to the

release of ammonia. Shown in Table 3 is the change in OD per minute that was

calculated from the average of three reactions.

Table 3. Induction of CHT Activity in *N. crassa*

Strain	NH$_4$ KCN	+ -	+ +	- -	- +
FGSC #2489		<0.1	10	<0.1	6.7
FGSC #9718		0.7	6.7	<0.1	7
FGSC #11824		<0.1	<0.1	<0.1	<0.1
FGSC #11825		<0.1	<0.1	<0.1	<0.1

The presence or absence of NH$_4$ (added as 25mM NH$_4$NO$_3$) or 2mM KCN during culture growth is designated above each column. Each lysate was assayed with 5mM KCN to measure the rate of cyanide degradation which is presented as ΔA_{540}/min x 1000 after addition of 0.5% picric acid. Show in this table is the average of triplicate experiments.

It appears that the presence or absence of nitrogen has no effect on expression, but the presence of cyanide during growth clearly causes induction of *cht* gene expression. The *cht* knockout strains had no detectable activity under any conditions.

A. nidulans grown with and without KCN showed similar KCN induction of nitrilase expression (not shown), but these assays were not analyzed quantitatively.

Measurement of Copper and Silver Effects

The electroplating bath waste water containing high concentrations of cyanide and either copper or silver were diluted 10-fold, as was a stock of KCN in metal-free water, bringing the concentrations of cyanide to approximately 100mM. The ability of each enzyme to degrade cyanide in the presence of copper and silver was measured over a 48 hour period (Figure 7). As a control, assays were run using KCN alone (Figure 7A). All of the cyanide in the control was degraded from these samples by 48 hours,

with *N. crassa* showing the fastest rate of cyanide degradation. Samples were also assayed in waste-water containing high concentrations of both cyanide and silver (Figure 7B). These assays were run using the same enzyme concentration as the KCN control assay. *A. nidulans* showed the least amount of cyanide degradation with ~30% of the cyanide remaining after 48 hours. The other three CHT enzymes degraded approximately 90% of the cyanide in the silver sample by 48 hours, with *N. crassa* degrading this amount after less than 5 hours. When the same amount of enzyme was used for the copper waste-water sample, very little degradation was observed (not shown). Shown in Figure 7C is the cyanide degraded from the copper sample when 10-fold more enzyme was added. For all four cyanide hydratases, 30% or more cyanide remained in the sample after 48 hours. This could be caused by the copper forming a complex with the cyanide, preventing degradation, or by copper inhibition of the enzyme. As before, the *N. crassa* CHT had the highest activity and degraded the most cyanide and *A. nidulans* showed the least, with greater than 60% of the cyanide remaining after 48 hours.
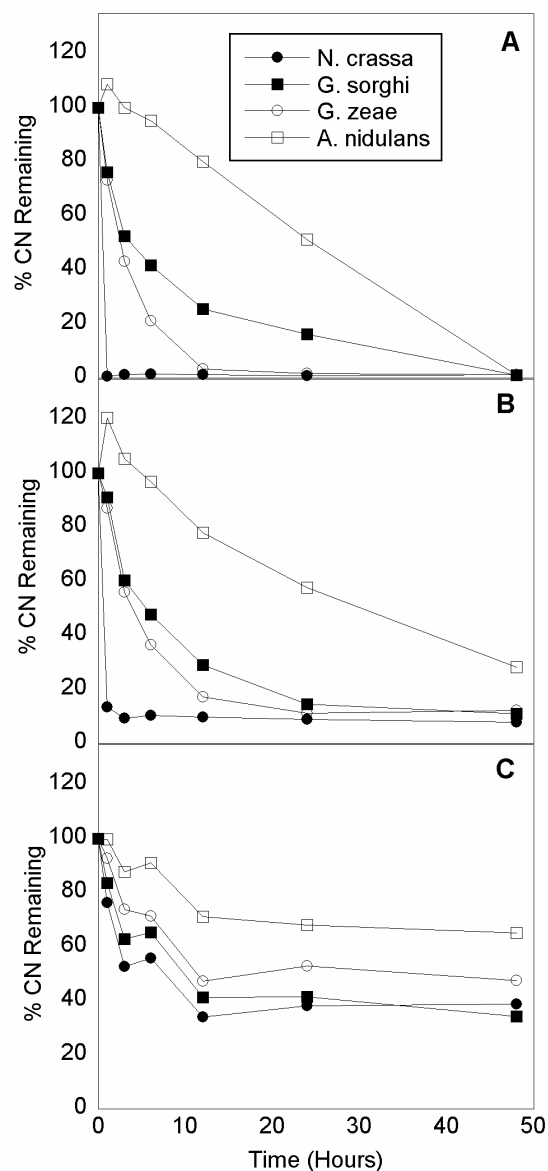
Figure 7: Ability of *N. crassa, G. sorghi, G. zeae,* and *A. nidulans* to degrade KCN (a), and cyanide in waste-water samples containing high concentrations of silver (b) or copper (c). Shown is the percent of cyanide remaining after assaying for periods between 0 and 48 hours. Each point is calculated relative to amount of cyanide at time point zero and is the average of duplicate experiments. Plot C using copper bath waste water was assayed using 10-fold more enzyme than (a) and (b).

CHAPTER IV

SUMMARY AND CONCLUSIONS

Due to the multitude of uses for cyanide in many industrial processes, the discovery and utilization of a cost effective and efficient process to eliminate the waste produced is necessary. Many industries, such as mining and pesticide production, use cyanide in large quantities. There are processes currently in use for cyanide destruction that use an oxidation reaction, but these can only be used effectively for certain types of cyanide-containing waste, such as slurry. There are also many enzymes that can degrade cyanide, but microbial nitrilases are the most promising candidates because they do not require any special conditions or cofactors for activity. Of these nitrilases, the cyanide hydratases (CHT) of fungi are more active than the cyanide dihydratases (CynD) of bacteria. The fungal nitrilases are also active at higher pH values than the cyanide dihydratases.

Activity at high pH values is important because cyanide-containing waste must be kept at pH 11 in order to keep the cyanide in solution. This, along with the presence of heavy metals in waste water, requires the enzymes used for remediation to have specific characteristics. The goal of my project was to find novel cyanide hydratase enzymes and characterize them based on pH profiles, stability, enzyme parameters, and ability to degrade cyanide in the presence of heavy metals. This information could eventually lead to production of an enzyme with the necessary characteristics to degrade cyanide in waste water.

In order to locate possible CHT enzymes, the protein sequence of *G. sorghi* was used to perform a BLAST search. Of the possible matches, the CHT of *N. crassa, A. nidulans,* and *G. zeae* were chosen. These were amplified and cloned into *E. coli*. A cDNA library was used for *N. crassa*, but genomic DNA was used from *A. nidulans* and *G. zeae* and introns subsequently had to be removed. Inactivity of the *A. nidulans* CHT led to the discovery of an incorrect assignment of introns in the database. After correction, all enzymes were expressed in *E. coli* with and without an amino-terminal 6-His purification tag. The tagged enzymes were purified by one-step purification using a nickel column and used for further characterization.

The pH profiles of the four recombinant fungal nitrilases (Figure 4) showed that *N. crassa* had the widest range of activity (at least half-maximal activity between pH 5.2 and pH 9) and *G. zeae* and *G. sorghi* displayed the narrowest range, with at least half-maximal activity relative to maximal rate between pH 6 and pH 8.5, approximately. *A. nidulans* displayed a relatively wide range of greater than 50% activity relative to maximal rate, but activity was below 80% for all but two pH values. The CHT of *Fusarium solani* was previously found to have maximum activity at a comparable pH 7.5 (6). Jandhyala et al. (2005) showed that CHT of *G. sorghi* is more tolerant of higher pH values when compared to the CynD of *B. pumilus* and *P. stutzeri*, but the two CynD enzymes appear to be more tolerant of lower pH values. These differences in pH tolerance are significant; applications generating cyanide waste water are routinely conducted at alkaline pH in order to reduce the formation of hydrogen cyanide gas, often above pH 10.

When comparing the thermal stabilities (Figure 5), there was a clear difference in the effect of higher temperatures on each cyanide hydratase. This is best shown by the enzyme activity remaining after 48 hours at 43°C. The CHT of *N. crassa* and *G. zeae* have higher relative activity at 48 hours than *G. sorghi* and *A. nidulans*. The high level of stability of *G. zeae* CHT contrasts to the enzyme's lack of stability in varying pH conditions. However, *N. crassa* continues to demonstrate it's versatility in diverse conditions.

Through correspondence with Prof. Trevor Sewell, it was suggested that the results obtained when attempting to determine kinetic values and the appearance of the graphs indicated substrate inhibition at high concentrations of KCN. Substrate inhibition occurs when high concentrations of substrate inhibit enzyme activity, often due to binding of the substrate at a non-active site. Substrate inhibition is not uncommon among the nitrilase group of enzymes (18, 36, 37). However, there is no evidence that this is due to allosteric effects, such as binding at a second, non-active site (19, 36). It is therefore presumably due to blocking of access to the active site by excessive substrate binding. Due to this inhibition, Michaelis Menten kinetic values were unable to be determined, but reaction rates were compared instead. *A. nidulans* and *N. crassa* had similar reaction rates at 20mM KCN, only slightly higher than *G. zeae;* however, that of *G. sorghi* was approximately 3-fold less. This is also true when comparing each enzyme's maximum reaction rate. *G. sorghi*, however, requires 20mM more cyanide than the other enzymes to reach its maximum rate of cyanide degradation. The Km and Vmax of other cyanide hydratases have been reported, but based on methods reported,

these values are not necessarily reliable. None of the assays went above the KCN concentration reported here to be the maximum activity of the enzyme before substrate inhibition was apparent. Barclay et al. (1998) reported the semi-purified CHT of *F. solani* to have a K_m of 4.7mM and V_{max} of 1.7μmol min^{-1} mg^{-1}, but their assay only went as high as 80mM KCN. Jandhyala (2002) only went as high as 30mM and found the K_m and V_{max} of the *G. sorghi* CHT to be 90mM and 4.4 mmol min^{-1} mg^{-1}, respectively. In light of findings reported here, this data is likely to be unreliable due to the low KCN concentrations used in the assays.

When comparing ability to degrade cyanide in the presence of heavy metals (Figure 7), *N. crassa* performed more efficiently than the other enzymes and degraded more cyanide at a faster rate. The CHT of *A. nidulans* had the least activity than the other cyanide hydratases when assayed with samples containing high concentrations of copper and silver. The CHT of *G. sorghi* has been shown to retain 75% or greater activity in the presence of cadmium, chromium, and iron (21). The enzyme does, however, lose 40% or more activity when mercury, lead, or zinc are present (21).

Other fungal nitrilases have previously been studied such as from *F. lateritium, F. solani, F. oxysporum* and *L. maculans*. Cyanide hydratase activity in all four of these organisms has been shown to be inducible by cyanide (6, 11, 43, 54). This work demonstrates that enzymes with similar activity and regulation are found in typical saprophytic strains of *N. crassa* and *A. nidulans* and not just the plant pathogenic fungi as previously thought. The amino acid sequence encoded by the *cht* of *N. crassa* is 72% and 82% identical to the sequences of *G. sorghi* and *F. lateritium*, respectively and that

of *A. nidulans* is the most distant of the group with about 60% identity to the other members. *F. solani* has been shown to degrade cyanide under acidic conditions (6) while the enzymes in this study are more active in the useful alkaline pH range.

Recently, the helical structure of *G. sorghi* and *N. crassa* were investigated (12, 53). Although the CynD from *Pseudomonas stutzeri* AK61 and *Bacillus pumilus* C1 are self-terminating, homo-oligomeric spiral of 14 and 18 subunits, the molecular weight of the CHT from *G. sorghi* is significantly higher (2-10 MDa) even though the subunit molecular weight is similar to that of the cyanidases (20, 41, 49, 53). Woodward et al. (2008) has shown that the CHT from *G. sorghi* is a left-handed spiral with interactions across the groove that differ from those seen in structural studies of the nitrilases from *P. stutzeri, B. pumilus,* and *R. rhodococcus* J1. Dent et al. (unpublished) showed similarities between the CHT of *N. crassa* and the nitrilase of *R. rhodococcus* J1, but there were two significant differences between the two. The nitrilase from *R. rhodococcus* J1 undergoes post-translational cleavage of the C-terminus, allowing formation of the helical homo-oligomer (46). The CHT of *N. crassa*, however, showed a helix with a greater diameter, which can accommodate the entire C-terminus without cleavage of the protein (12). This means that the CHT can form in the absence of proteolysis, but the nitrilase can only exist as inactive dimers without cleavage of the C-terminal amino acids (12). The other difference is in the helical twist operator, with *N. crassa* CHT having -66.67 and the nitrilase having -73.65 (12).

Comparisons of the many fungal nitrilases will help determine the best candidate for the remediation of cyanide-containing industrial wastewaters. Based on our results,

the CHT from *N. crassa* is our most promising candidate for this purpose. It shows high activity over a wide range of pH values, stability for at least 48 hours when incubated at temperatures ranging from 27-43°C, and a high rate of cyanide degradation. Due to new mutagenesis techniques being used in the lab, enzymes are being optimized to function at much higher pH levels than the wild type enzymes. With these CHT enzymes characterized and *N. crassa* CHT having promising characteristics, we hope to genetically optimize this enzyme for use in detoxification of cyanide-containing waste in the near future.

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