ISOLATION OF CYANIDE HYDRATASE MUTANTS FROM *GLOEOCERCOSPORA SORGHI* AT ALKALINE pH

An Undergraduate Research Scholars Thesis

By

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ABSTRACT

Isolation of Cyanide Hydratase Mutants from *Gloeocercospora sorghi* at Alkaline pH (May 2013)

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Cyanide is both a useful and dangerous chemical compound that serves as a crucial component in multiple industrial processes, including metal mining. The leaching process that utilizes cyanide ions to help separate target metals and increase mining yield is an industrial standard for chemical leaching. However, this method of ore extraction results in toxic cyanide waste that requires dangerous, costly, and potentially environmentally damaging remediation systems to degrade. As cyanide is a naturally occurring substance, several organisms contain enzymes capable of oxidizing cyanide into less toxic compounds. Despite the effectiveness of these proteins, they lack stability and functionality at the alkaline pH levels industrial cyanide is stored at. This project attempts to optimize the screening and mutagenesis methods in hopes of a isolating an alkaline tolerant mutant of cyanide hydratase, an enzyme originally found in the

fungus *Gloeocercospora sorghi*. This approach incorporates random mutagenesis of the target fungal gene using error-prone polymerase chain reaction and an *in vivo* picric acid assay that tests the activity of the mutant enzymes at target conditions. Experimentation was used to determine the ideal conditions for a screening method by testing the activity of the wild-type positive control at different reaction conditions. The final, optimized screening conditions for the high throughput assay combined a 50 μL aliquot of cell culture grown overnight in a 96 well plate with a 50 μL of 0.1 M CAPS buffered to pH 10.5. As screening continues, these conditions can be used to identify a viable, alkaline tolerant mutant. If such a mutant is identified, the molecule would be a strong bioremediation candidate for the metal mining industry and could lead to more efficient and environmentally friendly degradation of cyanide waste.

DEDICATION

To my family, especially my Grandmother who always has and will continue to serve as both an inspiration and role model of perseverance and humility.

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NOMENCLATURE

Weak Acid Dissociable (WAD)

Strong Acid Dissociable (SAD)

Chemical Leaching Agent (lixiviant)

Cyanide Hydratase (CHT)

Cyanide Dihydratase (CynD)

Cyanide Dihydratase from *Bacillus pumilus* ($CynD_{pum}$)

Cyanide Dihydratase from *Pseudomonas stutzeri* AK61 ($CynD_{stut}$)

Hydrogen Cyanide (HCN)

Parts Per Million (ppm)

Error-Prone Polymerase Chain Reaction (EP-PCR)

Plasmid DNA Preparation (miniprep)

Ampicillin Resistance (Amp^R)

Chloramphenicol Resistance (Cam^R)

CHAPTER I

INTRODUCTION

Cyanide

Cyanide is a well-known compound, both for its positive and negative qualities. While a useful industrial chemical and a crucial metabolite for some organisms, cyanide is also a fast acting toxin to most forms of life. This project is concerned with the removal of cyanide from contaminated waste-waters through the use of a modified fungal enzyme. The importance of safe and efficient industrial cyanide remediation, as well as information about this compound is discussed below.

Structural and Chemical Information

The cyanide molecule consists of a carbon atom triple bonded to a nitrogen atom and carries a single negative charge. Cyanide is a highly reactive compound, forming a variety of complexes with other elemental groups. These compounds include water soluble salts formed with members of the alkali metal group, commonly sodium and potassium. This type of cyanide is referred to as simple, soluble cyanide, which is often the basis for industrial mixtures (Needham 2003, MERG 2001). Cyanide can also form complexes with members of the transition metals; however, the intricacy of these complexes is variable, ranging from simple associations to complex binding arrangements, as well as weak to strong binding complexes (Needham 2003). The formation of these complexes contributes to the usefulness of cyanide in the metal mining industry. Cyanide associates with a number of organic and inorganic chemicals as a side group, which can form acid dissociable groups. These groups can be organized into Weak Acid

Dissociable (WAD) compounds or Strong Acid Dissociable (SAD) groups, and these types of compounds are defined by the amount of cyanide that becomes free as pH levels decrease (MERG 2001). Finally, cyanide pairs with hydrogen to form hydrogen cyanide (HCN), which can also be referred to as prussic acid or free cyanide. HCN is miscible in both inorganic and organic solvents and also evaporates at or above room temperature. Several common cyanide compounds, including HCN, emanate a bitter, almond-like smell, and most cyanide compounds are white or colorless (Needham 2003). HCN is a weak acid, with a pK_a value of 9.22 at 25^0C (MERG 2001).

Industrial Usage

Because of its high reactivity, cyanide compounds have been used in many industrial fields for myriad of purposes. The primary applications of cyanide are the production of plastics and extraction of metals in the mining industry, but there are many other industrial applications of cyanide, including electroplating, goods manufacturing, and steel hardening (MERG 2001). The metal mining industry utilizes a substantial portion of produced cyanide, consuming roughly 18 percent of total cyanide production (Logsdon 1999). The other percentage is used by an assortment of industries, including the manufacture of nylon precursors and other plastic products (MERG 2001). Cyanide is also used to "plate" one metal onto another, through a method referred to as electroplating. An example of this is the plating of gold and silver onto dishes and other such novelties (MERG 2001). Finally, cyanide can be used in the health profession to manufacture surgical dressings, anti-cancer preparations, and high blood pressure medications (MERG 2001). These different applications utilize cyanide's high reactivity, as well as its ability to form stable complexes with transition metals.

Cyanide is a compound used heavily in metal mining, but most importantly as a chemical leaching agent (lixiviant) in gold and copper extraction (Needham 2003). Cyanide first began its use in mining during 1887 in New Zealand, and has since become an integral part of gold and copper mining; cyanide continues to function as one of the cheapest and most efficient means of ore extraction (MERG 2001). Since most of the ore deposits located in mines typically contain a low concentration of gold as well as a mixture of sulfides and other compounds, lixiviants such as cyanide help to separate the desired ore from the other constituents while decreasing the percentage of contaminants (Needham 2003). Due to the poor grade of the ore of most modern mines, ore samples are often chemically dissolved with lixiviants and then physically processed through various means to remove unwanted compounds (Needham 2003). Because of the specificity of this application, chemicals that function well as a lixiviant are often difficult to identify, and despite over a hundred years of research, no chemical has proven to be a more cost effective lixiviant (EPA 1994). This is evidenced by cyanide's use in approximately 90 percent of the world's gold production (Needham 2003). Cyanide solutions used in the mining industry typically range from 100 to 500 parts per million (ppm) (Logsdon 1999). However, this amount is excess of the necessary amount for the reaction; this excess is to account for the cyanide lost during the extraction process through conversion into HCN gas, complex formation with other compounds, or oxidation to cyanate. Because cyanide is volatile at a neutral pH environment, cyanide waste-waters are typically kept at highly alkaline pH levels, approximately between 10 and 11, to minimize the amount of cyanide that dissipates as HCN gas (Needham 1999). The prevention of this volatilization not only serves as a safety precaution, but also as a process that can save money through the recycling of cyanide.

Heap leaching is a common technique used in heavy metal mining. The process begins by gathering the ore onto an impermeable pad and spraying the extract with the cyanide solution (Figure 1.1). Two electrochemical reactions that help to summarize the interaction of gold with cyanide are Bodlander's equation (Equation 1.1) and Elsener's equation (Equation 1.2) (EPA 1994). These equations are:

$$2Au + 4CN^- + O_2 + 2H_2O \leftrightarrow 2Au(CN)_2 + H_2O_2 + 2OH^- \quad \text{(Equation 1.1)}$$

$$4Au + 8CN^- + O_2 + 2H_2O \leftrightarrow 4\,Au(CN)_2 + 4OH^- \quad \text{(Equation 1.2)}$$

Both of these reactions lead to the formation of simple metal-cyanide complexes and release hydroxyl groups, which raise pH levels. Because of this, ore extraction cannot be done at extremely high pH levels. Since these reactions are also dependent on oxygen, the ore heaps must be aerated before or during the application of cyanide. Finally, the fully leached ore is separated, and the cyanide is discarded as waste. Cyanide waste typically results in the form of metal-cyanide complexes in a highly alkaline aqueous solution. However, despite making the waste less toxic, the metal complexes make it more difficult to remove the cyanide.

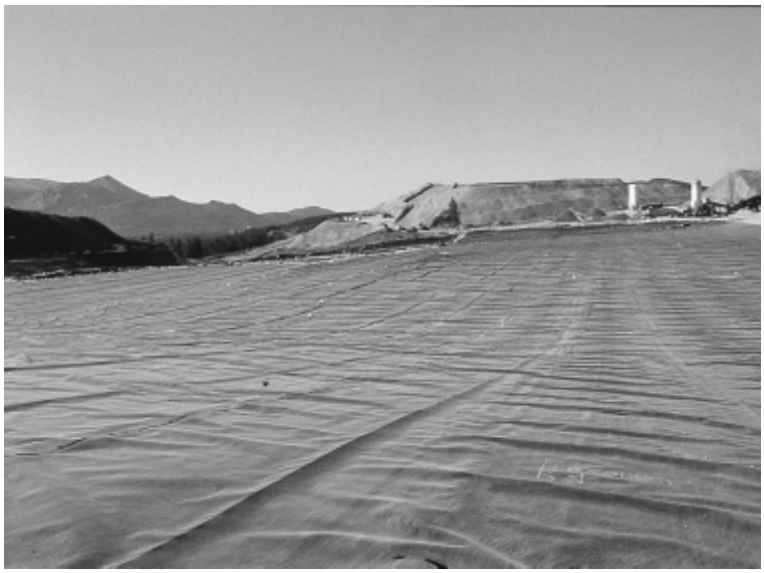

Figure 1.1 Construction of heap leaching pad at Pikes Peak, Colorado, USA (Logsdon 1999)

Toxicity

Cyanide is a highly toxic compound, and it derives this toxicity from the reactivity that is the root of its industrial importance. Cyanide binds to several key mitochondrial proteins that function in the electron transport chain. More specifically, cyanide binds to the iron containing cytochrome oxidase a_3 in Complex IV, inhibiting the protein's oxidizing ability and preventing the cell from using oxygen (Hamel 2011). Oxygen serves as a crucial electron acceptor in the oxidative phosphorylation process that takes place in mitochondria and produces the majority of the ATP in the cell. A failure of these essential enzymes leads to a disastrous chain of events and if untreated ends in death. With the failure of these enzymes, electrons cannot be passed down the electron acceptor pathway ending with the reduction of oxygen, which leads to the production of ATP. This failure to utilize the electron transport chain leads to oxygen deprivation in body tissues and eventually death through oxygen starvation, or hypoxia.

Cyanide poisoning can result from inhalation, ingestion, and absorption due to prolonged skin exposure. However, in an ore extraction facility, the threat of inhalation is greatest due to the formation of HCN gas from aqueous free cyanide. Table 1.1 illustrates the projected levels of cyanide toxicity due to inhalation. Cyanide also is toxic to wild flora and fauna in high concentrations, and this sensitivity must be accounted for when using cyanide in the metal mining.

Concentration (ppm)	**Response in Humans**
270	Immediately fatal
181	Fatal after 10 minutes
135	Fatal after 30 minutes
110 – 135	Fatal after 30 to 60 minutes or longer
45 – 55	Tolerated for 30 to 60 minutes without symptoms
18 – 36	Slight symptoms after several hours

Table 1.1 Average adult human toxicity levels to concentrations of HCN in air (MERG 2001)

Small amounts of cyanide are removed from the body by the liver; however, tissue of the central nervous, respiratory, and cardiac systems is especially sensitive to prolonged exposure to small doses (Hamel 2011). Cyanide poisoning can be treated by use of one of two antidotes, the

cyanide antidote kit and hydroxocobalamin (Hamel 2011). Speed is of the essence when diagnosing and treating cyanide poisoning, due to the fact that cyanide blocks the uptake of oxygen and this results in tissue death. Death of non-regenerative tissue, such as nervous tissue, leads to long term health problems if not death. The preferred method of decontamination depends on the type of exposure, namely due to the fact that each type of exposure causes a different set of health complications. In all instances however, the first step is to remove the afflicted individual from the contaminated area, which aids both the patient and the health care administrator. Also, cyanide causes a decrease in the amount of oxygen that the cell can use, so patients are typically treated with 100% oxygen to flood the body with oxygen and attempt to enhance the therapy (Hamel 2011). Finally, the antidote must be administered in order to begin counteracting the cyanide.

The antidote kit is comprised of three medications, amyl nitrite, sodium nitrite, and sodium thiosulfate (Hamel 2011). The amyl nitrite is inhaled, while the sodium nitrite and sodium thiosulfate are administered intravenously. The nitrites form methemoglobin that binds cyanide more preferentially than the cytochrome oxidase a_3, and the binding of cyanide with the methemoglobin forms cyanmethemoglobin (Hamel 2011). Methemoglobin binding to cyanide helps to decrease the effective amount of cyanide in the body, while thiosulfate is used in a transfer reaction, catalyzed by rhodanese, to form thiocyanate (Alexander 1989). This transfer reaction is the body's natural defense against cyanide. Thiocyanate is then filtered by the kidneys and removed from the body. The second of these antidotes is hydroxocobalamin, known as Cyanokit; it has been approved by the FDA, and treatment consists of one to two 5 g doses of hydroxocobalamin administered intravenously (Hamel 2011). Hydroxocobalamin binds to

cyanide and forms nontoxic cyanobalamin, which is filtered in the kidneys and removed in the urine (Hamel 2011). Both of these methods have helped to lower the death rate involved in cyanide related accidents.

Industrial Waste Treatment

Because of cyanide's role as an essential lixiviant, a myriad of ways to treat cyanide waste have evolved as a consequence. Despite the multitude of processes that can detoxify industrial cyanide, three general categories exist. The first way is the utilization of natural cyanide degradation; the second is the use of chemical additives that facilitate the breakdown of cyanide, and finally, the use of biological enzymes and microbes to degrade cyanide into less toxic products. The use of the second and third methods requires that the ore piles be rinsed with water (EPA 1994). Currently, several chemical and natural methods dominate the industry as standards for the reduction of cyanide waste into less toxic chemicals. However, research is being done to improve biological methods and create even more efficient ways to facilitate the removal of toxic cyanide waste. These methods of removal are controlled by the properties of the tailings pool, such as pH level of the waste, state of cyanide in solution, and temperature. The EPA maximum for cyanide concentrations in drinking water is 0.07 mg/L, and levels at or above this concentration not only damage humans, but it also affects aquatic life as well (Khodadadi 2005). Therefore, the goal of these methods is to lower the cyanide concentration of barren pools to a level similar to this.

Natural Degradation

Natural degradation, also called attenuation, is the dissociation of cyanide without the aid of chemical or biological additives. In aqueous solutions, free cyanide reacts with the aquatic environment to form HCN and enter the atmosphere; it is through this conversion that attenuation occurs, due to HCN's low boiling point and high pK_a. This phenomenon occurs in aqueous solutions with a pH lower than nine. WAD cyanide also can convert to HCN, but at much lower pH, around 4.5 (EPA 1994). The rate of attenuation is often controlled by the surface area of the leaching pool; a greater surface area contributes to a greater rate of attenuation because of the increased exposure to energy from the sun. However, natural degradation is often avoided, due to the toxic release of cyanide into the atmosphere and because cyanide is often recycled to reduce cost for the company. Furthermore, attenuation also increases the likelihood of environmental pollution, and makes the piles more susceptible to inclement weather. An attenuation unit is displayed below (Figure 1.2)

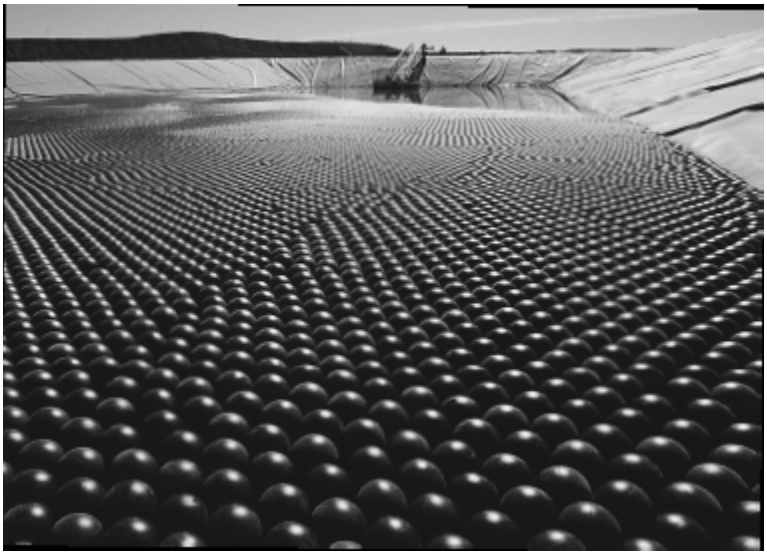

Figure 1.2 Open air cyanide waste containment with "bird balls" to protect avian wildlife (Logsdon 1999)

Chemical Treatment

There are various methods that utilize chemical additives to remove cyanide, and these methods currently are among the more popular protocols being used by the mining industry. These processes currently comprise the majority of industrial waste treatment methods due to the cost efficiency of these methods and from the amount of time that has been spent developing them. Some of the methods used by the mining industry currently include the use of sulfur, alkaline chlorination, the use of hydrogen peroxide, and AVR recovery.

When using sulfur to remove cyanide, sulfur dioxide combines with oxygen in the air to lead to the formation of cyanate and sulfuric acid (Equation 1.3). Copper serves as a catalyst in this process:

$$CN^- + SO_2 + O_2 + H_2O \rightarrow CNO^- + H_2SO_4 \qquad \text{(Equation 1.3)}$$

Because the tailing pool must stay at an alkaline pH, the sulfuric acid that is produced by the reaction must be neutralized. This is often done the use of lime or another strong base (EPA 1994). Several patents exist on various industrial methods for sulfur degradation, and these processes are referred to as the INCO Method and Noranda Method; both can be used towards wastewater treatment. Tests have shown that the INCO Method can reduce a cyanide solution of 1680 mg/L to 0.13 mg/L in a matter of 97 minutes (EPA 1993). These processes show inhibition at low temperatures and fail to remove toxic metals required to meet environmental regulations (EPA 1993). Therefore, other methods must be used to supplement INCO in order to fully decontaminate the water. A typical set up used for the INCO method can be viewed below (Figure 1.3).

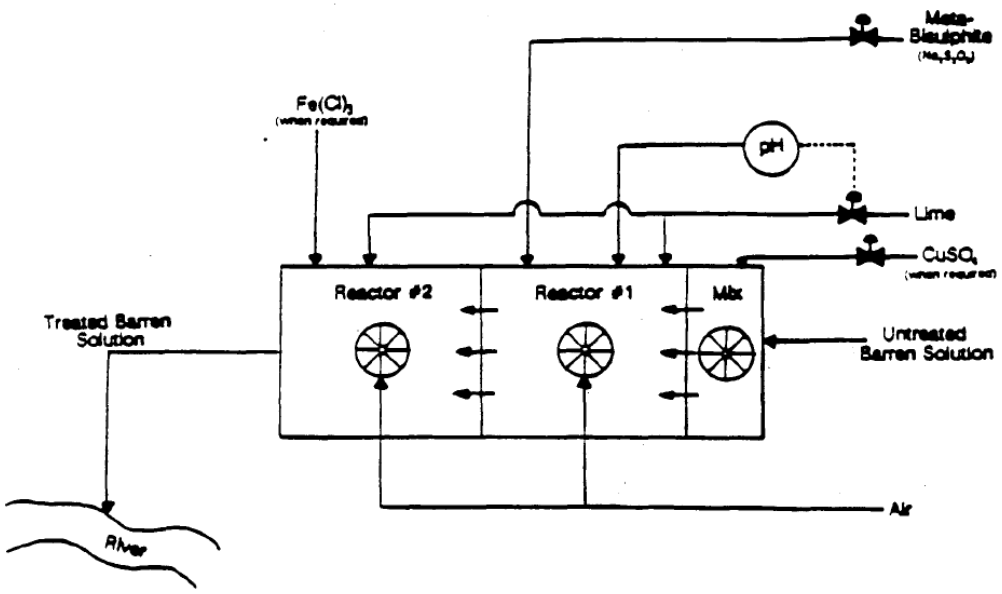

Figure 1.3 Diagram of typical two reactor INCO process (EPA 1993)

One of the oldest treatment methods still in practice is alkaline chlorination, where cyanide in solution is oxidized to cyanate. The reaction uses chlorine or perchlorate to oxidize cyanide to cyanate (Equation 1.4). The degradation reaction is described below:

$$CN^- + 2Cl^- \rightarrow CNO^- + Cl_2 \qquad \text{(Equation 1.4)}$$

Despite the longevity of this method, there are several reasons that alkaline chlorination is not ideal as a detoxification method on an industrial scale. These disadvantages primarily stem from the fact that this method is expensive, ineffective on cyanide complex waste, and produces a large quantity of sludge as by product (Khodadadi 2005). This process also allows chlorine, iron cyanide, and chloramines to remain in the waste. These chemicals prove toxic to aquatic wildlife and create additional decontamination issues.

Yet another process of cyanide destruction used in the mining industry is the use of hydrogen peroxide (H_2O_2) with a copper catalyst that breaks down cyanide into cyanate and water (Equation 1.5). Furthermore, the cyanate can be further broken down into carbonate and ammonia as displayed below (Equation 1.6).

$$CN^- + H_2O_2 \rightarrow CNO^- + H_2O \quad \text{(Equation 1.5)}$$

$$CNO^- + 2H_2O \rightarrow CO_3^{2-} + NH_4^+ \quad \text{(Equation 1.6)}$$

All three of these products are considerably less toxic than cyanide, and because of this, little additional treatment is required. This procedure must be conducted at a very alkaline pH (9 to 10) in order prevent the breakdown of the reactants, and this factor is controlled through the addition of lime. Finally, this method can break down both free cyanide and cyanide/metal complexes. However, hydrogen peroxide is a harmful substance and the equipment required for safe usage of can also increase the cost of cyanide destruction (EPA 1994).

Finally the use of Acidification-Volatilization-Recovery (AVD) methods are an industrial technique which act differently from most of the other prescribed methods. Instead of functioning at a high pH, the pH of the waste water is lowered to encourage the formation of HCN (Equation 1.7), which is then neutralized with sodium hydroxide (Equation 1.8).

$$CN^-(aq) + H^+(aq) \rightarrow HCN(g) \quad \text{(Equation 1.7)}$$

$$HCN(g) + NaOH(aq) \rightarrow NaCN(aq) \quad \text{(Equation 1.8)}$$

A major benefit of this method is the production of sodium cyanide, which can be recycled into the leaching process and save the company money. Lime is used to precipitate heavy metals in the water after treatment to meet environmental standards. However, this process is rarely used in industry primarily because it poses a huge health hazard because the reaction conditions

promote the formation of highly toxic HCN gas; in addition, the complexity of the process also discourages its role in industry and increases the cost (EPA 1994).

Biological Treatment

Research is being conducted to help engineer naturally occurring enzymes to function at alkaline conditions. These enzymes will hopefully be able to serve as a means of breaking down toxic, cyanide-laden wastewaters. The origin, mechanisms, and nature of these enzymes will be discussed at length later in the paper. Research is also being conducted to utilize specific plants to grow and remediate cyanide waste, while aiding in the extraction of heavy metals from solution and afflicted soil. The plants, *Helianthus annuus* and *Kalanchoe serrate* are being engineered to aid in the uptake of gold and copper from mining tailings and are supplemented with sodium cyanide and ammonium thiocyanate to promote greater uptake of the metals (Wilson-Corral 2011).

Cyanide in Nature

Cyanide is a naturally occurring substance, both being produced and decomposed by various organisms. Cyanide is produced by several plants and stored in the seeds, fruits, and roots of these plants, helping to deter the use of these organisms as a food supply. Several extreme examples include cassava and alfalfa, which have been known to produce cyanide poisoning in livestock and humans (Logsdon 1999). Furthermore, several species of centipedes, millipedes, insects, beetles, and butterflies have been known to utilize cyanide as a defensive mechanism against predators (MERG 2001). Several bacteria and fungi are able to break down cyanide

using enzymes to decompose these toxic compounds and utilize cyanide as a carbon and nitrogen source in the production of several essential metabolites.

While some species benefit from the presence of cyanide in the environment, high concentrations or prolonged exposure to low concentrations can be harmful to most life. In regards to terrestrial life, most interaction with human processing facilities is limited due to location and construction of metal mining plants. Due to this isolation, cyanide mining practices generally produce little effect on terrestrial animals. However, soil contamination is a true concern when dealing with cyanide pollution; cyanide degradation occurs at a slower rate in soil than in a natural body of water (Li 2001). Therefore, cyanide waste must be stored above ground, and these structures must be stable enough to withstand any inclement weather that the area would experience, so as to avoid leaks and spills. The main source of concern regarding birds and other aerial species is the exposure of these creatures to open tailings ponds and open air waste containment areas. This threat is reduced through the covering of these ponds as well as lowering the total available cyanide content of these ponds, specifically the WAD cyanide (Mudder 2004). Finally, due to the fact that most cyanide waste is contained in aqueous solutions, the group most naturally at risk to cyanide pollution is aquatic life. However, danger to this group of life can be greatly reduced by the correct storage and handling of toxic waste.

Improper waste containment or treatment can have catastrophic implications on the local environment. From 1975 to 2004, there were around 29 accidents in the mining industry, which averages out to approximately one major mining accident per year for this 30 year span (Mudder 2004). Of these 30 accidents, only 12 of them involved cyanide; this low incidence rate is

evidence of the strict regulation of cyanide waste by government agencies and companies (Mudder 2004). While cyanide exposure in the work place has a death rate of almost one death every two decades (Mudder 2004), cyanide accidents have been known to produce extensive environmental damage. Despite the low number of incidents and deaths, cyanide spills can be devastating to local environments. One such example is the Baia Mare Cyanide Accident in Romania, where a dam failure caused the contamination of the local rivers with cyanide waste, severely affecting both the aquatic and human life surrounding the rivers (Mudder 2004). This accident killed many fish and other members of the river ecosystem, while also affecting the food supply of riverside populations (Figure 1.4). Another example of a modern cyanide disaster is the spill that occurred at Gold Fields Ghana Limited. In this instance, dam failure resulted in contamination of local waters sources as far as 200 m of the site of leakage (Amegbey 2003). The spill was responsible for the death of local fish and also resulted in complaints of mild to moderate health problems in local citizens due to the water contamination (Amegbey 2003). Not only was the initial cyanide spill a source of distress for local citizens, but the remediation attempts also resulted in water contamination due to chlorine which affected local inhabitants (Amegby 2003). The adverse effects of these cyanide spills and the detoxification process support the call for new, improved procedures for cyanide detoxification, through methods such as engineered cyanide degrading enzymes.

Figure 1.4 After effects of the Baia Mare Cyanide Accident. Improper cyanide storage can have devastating effects on wildlife (Davids 2009)

Cyanide Metabolizing Enzymes

As cyanide is a naturally occurring substance, enzymes exist within organisms that contribute to the metabolism of cyanide. Found in several species of bacteria and fungi, these enzymes contribute to both the degradation of cyanide, and the use of cyanide in the synthesis of vital cell compounds. The effectiveness of these enzymes as bioremediation agents in several industries, including the mining industry, is currently being researched. Several factors that play a deciding role in determining the future of these proteins in the remediation of mining waste. They are: tolerance to high concentrations of cyanide, ability to dissociate the cyanide/metal complexes that form in industrial mining waste, and the enzyme's capacity to function without cofactors. These enzymes accomplish the breakdown of cyanide through several types of pathways, including reductive, oxidative, hydrolytic, and substitution pathways. These methods will be described in further detail.

Nitrilase Superfamily

The nitrilase super family is comprised of thirteen families of enzymes that can be categorized as amidases, N-acyltransferases and the nitrile degrading family that gave origin to the name of the super family (Brenner 2002). This paper will concern itself with the members of the nitrile degrading family, due to their possible application in industrial waste remediation. However, nitrilase proteins do demonstrate some unifying features. One such feature is the conserved Glu-Lys-Cys triad that is featured in the enzyme's active sites (Brenner 2002). Also, each member of the super family has noticeable sequence similarity to the other members of the family; in addition to the sequence similarity, the nitrilase superfamily also displays similar structural characteristics (Brenner 2002). Some of these similarities include conservation of subunit consistency and domain fusion (Brenner 2002). Nitrilase proteins consist of homodimeric domains and these domains are comprised of two αββα regions (Brenner 2002). Finally protein fusion between domains is also common in members of this superfamily, and these fusions can be used to categorize its members (Brenner 2002). However, one of the most useful tools in enzyme classification is the mechanism of reaction used by the enzyme.

Reduction Mechanism

The enzymes that typically utilize the reductive pathway of cyanide removal also function under anaerobic conditions, using a nitrogenase enzyme (Gupta 2010). Nitrogenase uses cyanide as a substrate to produce methane and ammonia (Equation 1.9), however this reaction requires several chemicals in addition to HCN, namely protons donated from ATP hydrolysis (Gupta 2010). The necessity of these cofactors detracts from this enzyme's usefulness as an *in vitro*

remediation agent. This mechanism has several inhibitors, including oxygen and other oxidizing agents. This type of enzyme is also found in soil organisms and fixes atmospheric nitrogen.

$$HCN + 6H^+ + 6e^- \rightarrow CH_4 + NH_3 \quad \text{(Equation 1.9)}$$

Hydrolysis Mechanism

Enzymes that oxidize free cyanide using a hydrolytic pathway are the cyanide hydratase (CHT) and cyanidase, formally known as cyanide dihydratase (CynD). CHT is found in several species of fungi, including *Neurospora crassa*, *Aspergillus nidulans*, *Gibberella zeae,* and *Gloeocercospora sorghi* (Basile 2008). CHT degrades cyanide into formamide (Equation 1.10), which can then be degraded into formate and ammonia using other enzymes. CynD is an enzyme that also requires no additional cofactors and is found in several bacterial species, including *Bacillus pumilus* and *Pseudomonas stutzeri* (Gupta 2010). This enzyme functions to degrade cyanide into formate and ammonia, as displayed below (Equation 1.11) (Gupta 2010). Research is currently targeting this class of proteins as a means for bioremediation, based off the fact that they degrade cyanide without the need of additional cofactors (Jandhyala 2005). However, these enzymes do fail to function at alkaline pH levels and at high concentrations of cyanide; the current goal of research is to attempt to engineer these enzymes so they function under the harsh conditions experienced in the tailing ponds.

$$HCN + H_2O \rightarrow COH_3N \quad \text{(Equation 1.10)}$$

$$HCN + 2H_2O \rightarrow NH_3 + HCOOH \quad \text{(Equation 1.11)}$$

Oxidation Mechanism

Three enzymes use an oxidative pathway of cyanide degradation, and these proteins are cyanide monooxygenase, cyanase, and cyanide dioxygenase. However, between these three enzymes, there are only two mechanisms of oxidative destruction. One is used by cyanide dioxygenase, and the other is shared by cyanide monooxygenase and cyanase (Gupta 2010). Cyanide dioxygenase uses oxygen and NADH to break down cyanide into carbon dioxide and ammonia (Equation 1.12). On the other hand, cyanide monoxygenase and cyanase use oxygen and NADH to produce cyanate and water (Equation 1.13) (Gupta 2010). Because of the critical role of NADH in the reaction, this enzyme is difficult to isolate outside of a cell and therefore makes it difficult for this type of enzyme to serve as an *in vitro* bioremediation candidate.

$$HCN + O_2 + H^+ + NADH \rightarrow CO_2 + NH_3 + NAD^+ \qquad \text{(Equation 1.12)}$$

$$HCN + O_2 + H^+ + NADH \rightarrow HOCN + NAD^+ + H_2O \qquad \text{(Equation 1.13)}$$

Transfer Mechanism

In addition to degrading cyanide, several enzymes allow for the uptake and integration of cyanide into the cell by using it as an additional nitrogen source or to allow the organism to remediate the toxic effects of cyanide within its own body. These mechanisms are carried out by the rhodanese and mercaptopyruvate sulfurtransferase enzymes. Rhodanese is a highly conserved protein and can be found in a variety of organisms. It uses thiosulfate and cyanide to produce sulfite and thiocyanate by transferring a sulfur containing group to cyanide (Alexander 1989). This transferase is believed to have evolved as a mechanism for cyanide detoxification and actually serves as the major natural means of cyanide detoxification in mammals (Alexander 1989). Mercaptopyruvate sulfurtransferase is an enzyme that also helps to detoxify cyanide by

converting it to thiocyanate through the transfer of a sulfur group. However, this enzyme first accepts a sulfur group from the sulfur source (Equation 1.14) and then transfers the sulfur group to cyanide ion (Equation 1.15). Mercaptopyruvate sulfurtransferase is also a widely conserved protein, although it was first discovered in the parasite trypanosomatid *Leishmania major* (Gupta 2010).

$$HSCH_2COCOO^- + E \rightarrow CH_2COCOO^- + ES \qquad \text{(Equation 1.14)}$$

$$ES + CN^- \rightarrow E + SCN^- \qquad \text{(Equation 1.15)}$$

Nitrilase Protein Research

Nitrilase proteins are a class of enzymes that remove cyanide by either converting the CN functional group into less toxic compounds, such as formamide or ammonia, or transferring the CN group onto another compound. While the various pathways of degradation and transfer are detailed above, two classes of enzymes are important enough to be explored more closely. These enzymes are CHT and cyanide dihydratase (CynD). In the search for suitable candidates for use in mining bioremediation, these two classes of proteins are obvious choices due to the fact that they require no additional cofactors. These enzymes are similar in structure and function; however, the mechanism of the reaction they catalyze and the end products of their respective reactions are different. Our lab is currently investigating both enzymes, but much more research has been conducted on two bacterial CynD. Therefore, this study will draw from the techniques used and reference information acquired through that previous research. The purpose of this project is to determine screening conditions capable of isolating alkaline tolerant mutants from CHT from *G. sorghi* to be capable of functioning at a highly alkaline pH.

Structure of a Related Cyanide Dihydratase

While research and information in regards to the structure of the CHT from *G. sorghi* is limited, more research has been conducted on the similar enzyme nitrilase protein, AK61, originating from *Pseudomonas stutzeri*. Therefore, these findings can be used to help portray the general structure of CHT. Results from tests, such as SDS-PAGE gel, gel filtration, and especially electron microscopy, have lead researchers to believe that most nitrilase proteins exist as homo-oligomers (Sewell 2003). The exact number of subunits utilized by the fully functional protein is dependent on the species and type of nitrilase (Sewell 2003). The enzyme that was isolated from AK61was shown to be a 14 unit enzyme complex. Homology modeling and structural analysis was used to analyze the peptide sequences of three related enzymes originating from three separate sources (*P. stutzeri*, *B. pumilus*, and *G. sorghi*) and this analysis identified a conservation of peptide sequences in multiple regions (Sewell 2003). Below is a view of $CynD_{pum}$ visualized using PyMOL visualization software with data provided by a personal communication with Dr. Sewell on 12/10/2012 (Figure 1.5 and 1.6). While this is not the structure of CHT, the two enzymes are similar and these pictures are meant to provide a visualization of the nitrilase super family. A projection of CHT is also shown below (Figure 1.7); this model was constructed based off results from scanning electron microscopy (Woodward 2008).

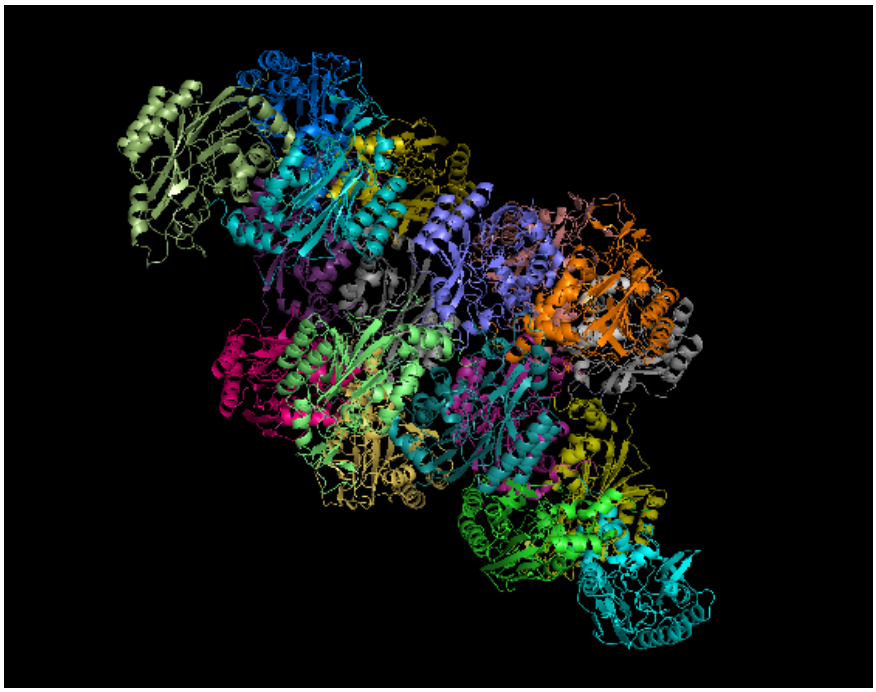

Figure 1.5 View of quaternary structure of CynD$_{pum}$ at pH 5.4, visualized using PyMOL

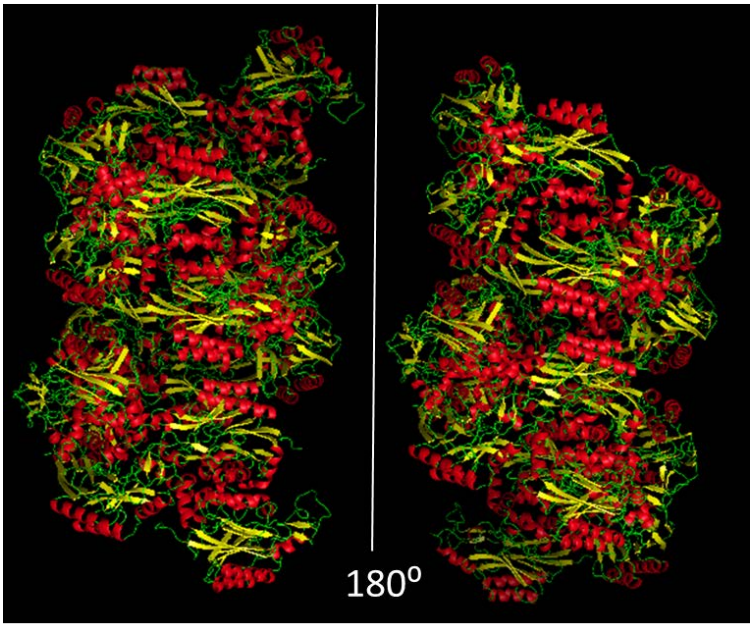

Figure 1.6 View of opposite sides of the CynD$_{pum}$ enzyme with secondary structures highlighted, visualized with PyMOL

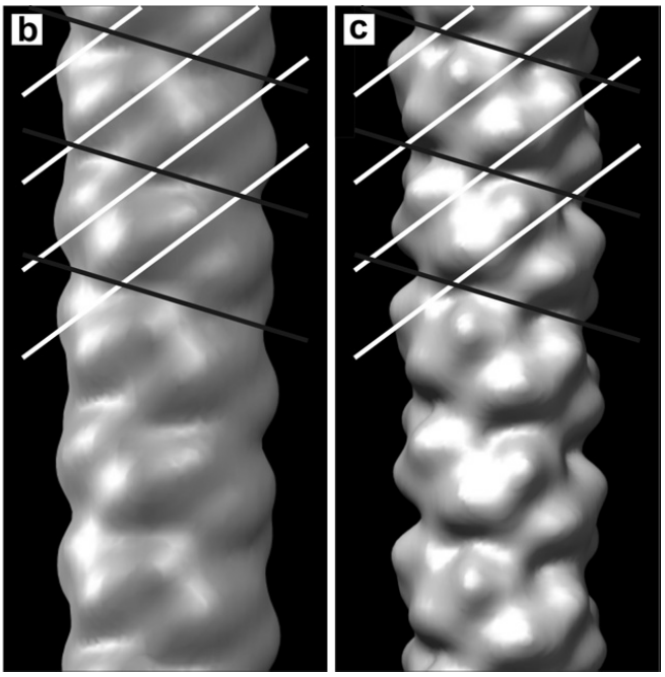

Figure 1.7 (b) Metal shadowed and (c) cryo-reconstruction approximations of CHT structure (Woodward 2008)

Cyanide Dihydratase Protein Engineering

The work done on this project is based off research previously conducted with two bacterial CynD enzymes. The bacterial origins of these enzymes are *P. stutzeri* and *B. pumilus*; the aim of the previous research was to produce an alkaline tolerant mutant CynD capable of serving as a bioremediation agent for the mining industry. That work originally focused on the CynD that originates from *B. pumilus*. The lab combined error-prone polymerase chain reaction (EP-PCR) with a high throughput screening strategy in order to identify mutations that conferred alkaline tolerance. Of the 2,000 mutants screened, two mutant candidates were isolated, referred to by the author as C5 and H7 (Wang 2012). The two identified mutants contained three mutations apiece which helped to confer alkaline tolerance (Wang 2012). Variations of the identified mutations were isolated and combined into double mutant groupings designed to test the effect

that each mutation had on the mutant enzyme. The C5 mutant displayed only maximum alkaline activity in the triple mutant combination, indicating that the acquired alkaline tolerance was gained due to the combined effect of all the mutations, rather than one mutation in particular (Wang 2012). On the other hand, one mutation in the H7 mutant displayed similar levels of alkaline tolerance in comparison to the H7 triple mutant, suggesting that this one mutation (E327G) was responsible for the majority of the pH tolerance (Wang 2012). These mutations were believed to confer an increased stability to the enzyme at higher pH levels, which then resulted in an increased alkaline tolerance (Wang 2012). Evidence that supports this belief can be observed in negative stain transmission electron microscopy of these mutants, where the images suggest that these mutations contribute to a delayed denaturation of the CynD (Wang 2012).

Further work was done by our lab with the bacterial CynD enzymes originating in both *P. stutzeri* and *B. pumilus*. For the CynD from *B. pumilus* ($CynD_{pum}$), random mutagenesis screening was performed to search for mutant enzymes with a higher activity level than the wild-type. This resulted in three mutations that were isolated due to increased alkaline activity of the enzyme *in vivo* (Abou-Nader 2012). Two of the mutations (K93R and D172N) were shown to increase the thermo-stability of the enzyme by several folds; in addition, D172N also increased the enzymes substrate affinity at cytosolic pH levels (Abou-Nader 2012). A third mutation (E327K) also led to a less significant increase in stability at neutral pH (Abou-Nader 2012). However, none of these mutations displayed any notable effect on the enzyme at alkaline pH levels.

After these mutants were identified, research on the different C-terminus tail regions of the *P. stutzeri* and *B. pumilus* CynD enzymes was conducted. While the two enzymes have analogous sequences, sharing nearly 76% similarity, one major region of difference is the C-terminus region (Abou-Nader 2012). Tests were conducted to examine the importance of this region, which included deleting the respective C-termini and swapping these regions on the two enzymes. First, the $CynD_{stut}$ showed inactivation after it sustained any deletion at or before the 310^{th} residue (Abou-Nader 2012). Further research indicated that this region was necessary for enzyme activity, due to the speculation that this domain interacted with the C surface of the enzyme and helped to position residues important for catalytic activity (Abou-Nader 2012). However, the C-terminus of $CynD_{pum}$ did not display as crucial a role as the analogous region of $CynD_{stut}$; the deletion did not affect catalysis, but did lead to decreased stability (Abou-Nader 2012). The C-terminal deletion in the mutant $CynD_{pum}$ allowed the enzyme to retain catalytic activity but caused destabilization; approximately 90% of its function was lost after 15 minutes of incubation at 38° C (Abou-Nader 2012). Research was also conducted on the effect of interchanging the tail regions between the two enzymes.

The replacement of the C-terminus region of $CynD_{stut}$ with the analogous tail region from the $CynD_{pum}$ resulted in an inactive enzyme (Abou-Nader). However, when the C-terminus of $CynD_{stut}$ was added to $CynD_{pum}$, stability actually increased (Abou-Nader 2012). There was no observable effect on the activity of the enzyme at cytosolic pH, and this tail swap actually increased stability of the enzyme at alkaline pH. The $CynD_{pum}$-$CynD_{stut}$ hybrid displayed 100% activity at pH 9, and 40% activity at pH 9.5, showing it to be an alkaline tolerant enzyme (Abou-

Nader 2012). Results like these support the effectiveness of protein engineering and provide evidence that an effective bioremediation agent can be produced from this class of enzymes.

G. sorghi Cyanide Hydratase Enzyme Characteristics

CHT is a solid candidate as a bioremediation agent for several reasons. The first is that it functions at a high catalytic rate in comparison to other enzymes. The related CynD from *B. pumilus* functions with a Vmax of 0.097 ± 0.011 $\frac{mmol}{(min)(mg)}$ and the CynD for *P. stutzeri* AK61 has a Vmax of 0.10 ± 0.016 $\frac{mmol}{(\text{min})(mg)}$ (Jandhyala 2005). Under the same conditions, CHT displayed a Vmax of 4.4 ± 1.5 $\frac{mmol}{(\text{min})(mg)}$ (Jandhyala 2005). With the exception of lead and mercury, CHT does not display levels of inhibition greater than approximately 25% and is relatively stable at room temperature (Jandhyala 2005). However, the greatest problem with CHT in regards to its use for bioremediation is its lack of stability at pH levels greater than 8 (Jandhyala 2005). Since most industrial cyanide waste is typically kept at pH levels greater than 9 to avoid the formation and consequent evaporation of the toxic HCN. Therefore, any feasible candidate for industrial bioremediation must show functionality and stability at a pH greater than 9.

Protein Engineering

Two modern methods of protein engineering exist, the directed evolution method and rational design method. Both of these approaches have potential benefits and disadvantages that benefit specific types of research and development. These short-comings and advantages will be discussed in detail, along with a brief description of the methods themselves. Finally, an overview for the method being developed in this project will be given.

Directed Evolution

In the directed evolution technique, mutagenesis methods are used to create random mutations in the designated gene sequence. Techniques such as EP-PCR act to provide slight mutations to the target gene, in hopes that these mutations will lead to the desired phenotype. The gene must then be transformed into bacteria and grown with a selective marker. The single colonies from the transformation are then tested through a high throughput screening method under the desired conditions. The desired mutants are then subject to the same methods and continually improved until mutants with the desired quality are produced. A diagram of the process typically used in directed evolution is displayed below in Figure 1.8. Directed evolution has some distinct advantages. The first is that directed evolution does not require any prior knowledge of the enzymatic structure, how this structure relates to the function of the molecule, or the enzymatic mechanism (Chen 2001). Also, this technique tests for function rather than specific structures, which is beneficial for industrial production of new, efficient enzyme products. Finally, directed evolution can also lead to interesting new discoveries, such as new functions in target molecules that arise due to the random mutagenesis (Chen 2001). However, this technique is not without its short-comings. One such downside is that this technique does not typically lead to drastic changes with the enzyme, but rather, simulates the rapid effect of evolution by inducing small changes to the protein. Therefore, this aspect of the technique makes it inefficient to search for rapid, large scale improvement of the target molecule. Also, due to the random nature of the mutagenesis, the technique can require large amounts of testing to achieve the desired result. Finally, directed evolution requires a sensitive and efficient method of screening mutants, which may not be a feasible option for some enzymes (Chen 2001).

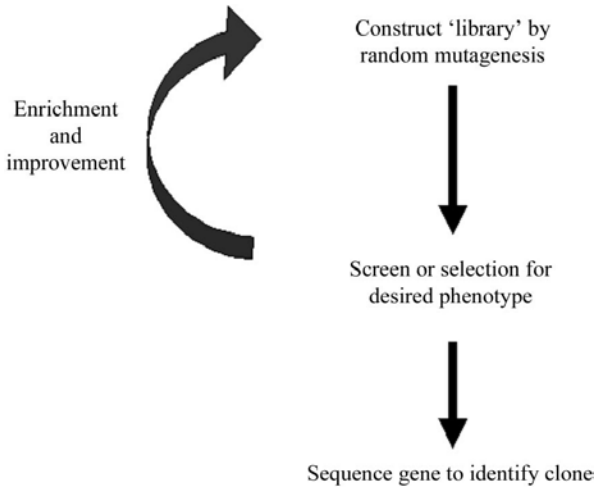

Figure 1.8 Diagram of technique used in directed evolution (Hart 2006)

Rational Design

In rational design, the 3-D structure of the protein is used to aid researchers in the identification of key residues and guide site-directed mutagenesis in an attempt to optimize the protein. This 3-D structure can be determined by both experimental and computational methods; however, this technique typically uses a combination of both. Experimental techniques include methods such as Nuclear Magnetic Resonance (NMR) spectroscopy and X-ray crystallography. Data gathered from these techniques is sequenced and assembled in order to define a structure. The defined structure can be visualized using software such as PyMol. From the visual data given of these structures, researchers can identify what they believe to be crucial residues and ideal sites for mutagenesis. Another type of structure determination that is being developed is software that can determine structure from primary sequence. There are two major types of structure prediction methods being developed, *de novo* and homology modeling. Homology modeling uses the protein class and sequence data to compare the unknown protein's primary sequence to

previously determined structures. *De novo* prediction, on the other hand, uses a scoring function to calculate structural conformations based off of the primary sequence and is purely a computational technique. Rational design can be used to study the specific interactions of protein residues and give insight to the impact of conserved residues. Rational design also can be used to introduce mutations at key sites that might not have been possible with random mutagenesis (Chen 2001). Yet, this method is not without its pitfalls. The first of these limitations is the need for accurate structural data in order to conduct the research. The experimental techniques are typically very intricate and require a lot of time and effort to generate reliable results. Also prediction software is not accurate enough to generate reliable models to base predictions off of. The lack of structural data can make the directed design process unfeasible for some proteins. In addition, the enzymatic mechanism must also be known so that the directed mutations conserve the catalytic activity. But as prediction software continues to develop and protein structure databases continue to grow, this technique will become more efficient and applicable (Chen 2001).

Objective of Research

This project will develop techniques that are similar to those used in directed evolution and are capable of both generating and isolating a functional, alkaline tolerant mutant of the *G. sorghi* CHT. These techniques include random mutagenesis and high throughput screening to identify and isolate mutant enzymes with an increased activity at an alkaline environment. The project will use EP-PCR as a method of random mutagenesis, and then transform the mutated gene into a bacterial vector using electrophoresis *in vivo* transformation. Then the mutants will be screened using a picric acid assay optimized to test for heightened enzyme activity at alkaline pH

levels. These methods will be detailed later in this paper. Eventually, this project aims to use these methods to generate mutant enzymes that display alkaline tolerance. Such mutants could serve as bioremediation agents for the mining industry.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains and Plasmids

This project uses multiple strains of *Escherichia coli* bacteria in order to express, clone, and reproduce the cyanide hydratase (CHT) enzyme found in *Gloeocercospora sorghi*. These strains are described below in Table 2.1. Cultures were grown in LB broth and supplemented with 100 μg/mL ampicillin and 25 μg/mL chloramphenicol when necessary. Plasmids used in this study are described in Table 2.2. Vector maps for the pBS (+SK) and pBC (+SK) plasmids can be located in Appendix A.

Bacterial Strains	Description	Reference
MB3436	Heat-Shock competent cells	Lab stock
MB3635	Strain containing *G. sorghi* CHT wild-type gene	Lab stock
MB4091	Electro-competent cells containing DH10B pKD46 with *red* recombinant genes	Lab stock
MB4105	Strain containing pBC-ccd cloning vector	Lab stock
MB4599	Strain containing pBS (SK+) cloning vector	Lab stock
MB5436	Strain containing wild-type *cht* gene in pBS (SK+)	This work
MB 5438	Strain containing wild-type *cht* gene in pBC (SK+)	This work

Table 2.1 List of bacterial strains used in research

Plasmid	Description	Reference
pBC (SK+)	*E. coli* cloning vector, 3.4 kB and Cam^R	Stratagene
pBS (SK+)	*E. coli* cloning vector, 3.0 kB and Amp^R	Stratagene

Table 2.2 List of plasmids used in research

Plasmid DNA Preparations

Plasmid DNA preparation (miniprep) was used to isolate bacterial DNA, which in turn allowed the plasmid DNA to be manipulated and utilized as needed. This project used two different kinds of minipreps. General minipreps were used to lyse the bacterial cells and isolate the cytosolic contents. Phenyl-chloroform minipreps were used to precipitate bacterial DNA and remove other molecules from the cytosolic extract. In order to prepare the pBS and pBC vectors to accept the target *cht* gene, the *Xba* and *Xho* restriction endonucleases were used. These endonucleases were used to cleave the plasmids in the multiple cloning site and allow for recombination. The endonucleases were purchased from New England Biolabs (Boston, MA). The positive selection vector used for the generation of mutant libraries was pBC-ccd, found in strain MB4105. After the plasmid restriction digestion, the endonucleases were heat-killed, and the DNA was precipitated with an equal volume of isopropanol, washed with 200 μL of 70% ethanol solution, and suspended in 15 μL of MQ H_2O. The concentration of the vector DNA was determined measuring the absorbance at 260 nm using the Nanodrop ND-1000 spectrophotometer.

Isolation of Alkaline Tolerant Mutants

The objective of this research was to use error-prone PCR (EP-PCR) to generate random mutant libraries and develop a high throughput screen for these libraries that can identify mutants with increased alkaline tolerance. Similar techniques for have been employed on analogous enzymes, such as cyanide dihydratase (CynD), with success in the past (Abou-Nader 2012, Wang 2012). The mutagenesis technique used in this experiment was EP- PCR, and the screening method used was a picric acid assay, which detects free cyanide through a cyanide dependent colorimetric change. A graphic model of this procedure is located below (Figure 2.1), and the techniques used in this project are also further detailed below.

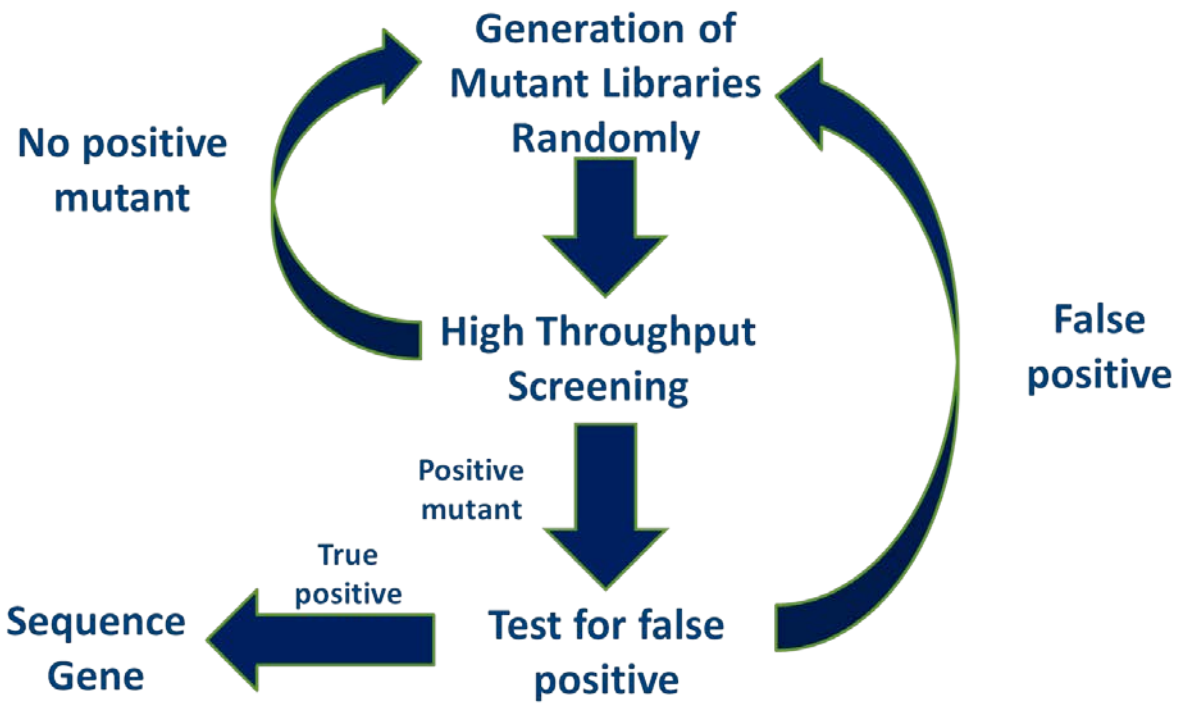

Figure 2.1 Graphic explanation of project flow in attempt to isolate alkaline tolerant mutants.

Error-prone PCR

Polymerase chain reaction is a technique that amplifies a specific gene sequence, which can then be used for analysis or DNA cloning. Error-prone PCR (EP-PCR) is a technique that induces random mutations into the gene sequence that it amplifies. This is accomplished through the use of a low fidelity polymerase and the addition of manganese, which raises the mutation rate. The procedure used in this research followed the EP-PCR mixture outlined in *Rapid Generation of Mutant Libraries* (Abou-Nader, 2010). Taq 2x Mixture was purchased from New England Biolabs (Boston, MA), and the primer sequences used to amplify the CHT sequence from p3536 were the -60M13F and -60M13R primers (Table 2.3). For each PCR reaction, 25 μL of Taq 2x Mixture was combined with 100 ng of each primer (1 μL a piece), 1 μL of the plasmid DNA containing the *cht* gene, 1 μL of 10 mM $MnCl_2$, 5 μL of 25 mM $MgCl_2$, and 16 μL of MQ H_2O to complete a 50 μL setup. The EP-PCR reaction followed the sequence outlined in Table 2.4. After the EP-PCR was complete, the DNA was precipitated with an equal volume of isopropanol, washed with 200 μL of 70% ethanol, and suspended in 15 μL of MQ H_2O. The concentration was then measured the absorbance at 260 nm using the Nanodrop ND-1000 spectrophotometer.

Primer Name	**Primer Sequence**
-60M13F	5'-GCGAAAGGGGGATGTGCTGCAAGG-3'
-60M13R	5'-CACTTTATGCTTCCGGCTCGTATG-3'

Table 2.3 Sequence of Primers used in EP-PCR

Step Name	Temperature	Duration	Repeats
Initial Denaturation	**95^{o}C**	**3 min**	**1x**
Denaturation	**95^{o}C**	**30 sec**	**25x**
Annealing	**55^{o}C**	**1 min**	
Extension	**72^{o}C**	**1 min 30 sec**	
Final Extension	**72^{o}C**	**7 min**	**1x**
Hold	**4^{o}C**		

Table 2.4 Outline of reaction cycle used in Error-prone PCR

Recombinant DNA Methods

Construction of the MB5436 strain was completed using a combined approach of *in vitro* recombination and heat shock transformation. The wild-type *cht* gene was combined with the pBS cloning vector using a 1:4 ratio and then ligase was added to the mixture to join the two fragments. The recombined plasmid was then transformed using 50 μL of heat-shock competent cells (MB3436) and 4 μL of ligated DNA. After the DNA was added, the cells were left on ice for ten minutes. After ten minutes, the cells were transferred to the 42°C heat well for 90 seconds. Next, the cells were placed back on ice for two minutes and recovered in LB broth for 45 minutes at 37°C. Finally, the recovered cells were plated on LB agar plates supplemented with ampicillin, and the individual colonies were grown and tested for CHT activity. This

recombinant strain served as a source for the *cht* gene, which would then be amplified through the use of EP-PCR.

The generation of random mutant libraries was accomplished by the use of an *in vivo* cloning technique, which utilized electro-competent *E. coli* cells (MB4091). The MB4091 cells were thawed on ice; then the mutated *cht* gene and pBC vector (MB4105) were introduced into the MB4091 cells in a 1:4 vector to gene ratio, and the cell DNA mixture was inoculated for 20 minutes on ice (Abou-Nader, 2010). After 20 minutes, the mixture was transferred to a chilled 2 mm electroporation cuvette, and 2500 volts was applied for 5 ns to the cuvette. The cells were recovered in LB broth for 45 minutes at 37°C, and then plated on LB agar plates supplemented with chloramphenicol. The plates were grown at 37^0C overnight, and the individual colonies were picked and inoculated in sterile 96 well-plates filled with 200 μL LB broth supplemented with chloramphenicol. Transformations typically produced between 85 and 110 colonies per plate for five plates, generating an average of between 500 and 650 transformant cells. Of those transformed colonies, approximately 300 colonies were plated. In each plate, 94 wells were inoculated with the mutants, and the other two wells contained either a wild-type enzyme or a MB4105 negative control. A graphic depiction of this technique is shown below (Figure 2.2).

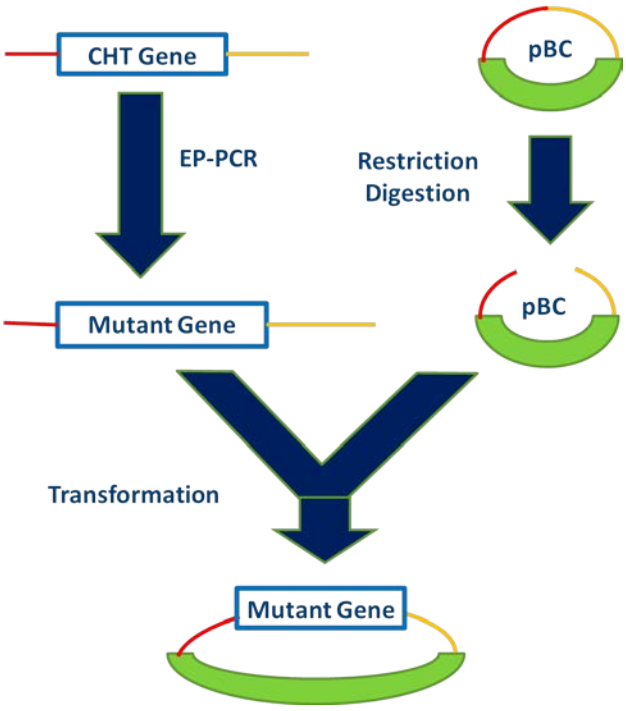

Figure 2.2 Construction of mutant libraries through use of *in vivo* cloning.

High Throughput Mutant Screening with Picric Acid

A sensitive, high throughput screen was required for the isolation and identification of mutants with increased alkaline tolerance. In the past, our lab has used picric acid as a colorimetric indicator for cyanide in screening assays with the related CynD enzyme (Wang 2012). This research used a screening method similar to the protocol used with CynD; however, this approach has not been applied to CHT screening, and therefore, the ideal testing conditions needed to be determined and optimized before mutant screening could begin. Picric acid is a compound that typically has a very bright yellow color, but in the presence of cyanide, it turns orange to red in a cyanide concentration dependent manner. Because of its sensitivity to cyanide, picric acid will be used as a colorimetric indicator for the activity of the CHT mutants.

Due to its reactive nature, a dilute solution of picric acid (1.2% Weight/Volume) was mixed in a 1:1 ratio with 0.5 M sodium carbonate.

The screening conditions were determined through experimentation in two different procedures. The initial analysis tested variable pH levels and time intervals to determine conditions that would be capable of identifying alkaline tolerant mutants. These conditions were determined using MB5438 cells grown as an overnight culture tube. The constants in the determination experiment were the concentration of cyanide (20mM), volume of the reaction before the addition of picric acid (100 μL), and the time of reaction (30 minutes). The variables were the pH of the environment (8 through 11 in increasing intervals of 0.5) and the volume of cells used in the screen (10 through 50 μL in increasing intervals of 10 μL). The conditions for mutant library screening determined this screen used 25 μL of cells, 25 μL of 0.1 M CAPS buffer at pH 11.0, and 50 μL of 20mM cyanide/0.1 M CAPS buffer (pH 11.0). The reaction was allowed to run for 30 minutes, and then the reaction was stopped with the addition of equal volume picric acid mixture. The picric acid was then inoculated at 65°C for 20 minutes, and the colorimetric change was noted.

The second determination of screening conditions used constant cell culture volumes (50 μL), cyanide concentrations (20 mM), and overall reaction volume before the addition of picric acid (100 μL). The variables measured were pH of the environment (pH 10.0, 10.5, and 11.0) and the reaction time interval (10 to 45 minutes in increasing intervals of 5 minutes). The conditions determined by testing used 50 μL of cells grown in well plates and 50 μL of 20 mM cyanide/0.1 M CAPS buffer (pH 10.5). The reaction was run for 10 minutes and then stopped with picric

acid. The assay was inoculated for 20 minutes at 65°C, and the assay was assessed for colorimetric changes that indicated viable, alkaline tolerant mutants. These conditions were selected because the wild-type enzyme failed to show substantial cyanide degradation under these conditions. It is presumed that a small increase in the pH tolerance of a mutant would yield a positive reaction, which would be easily distinguishable from the decreased activity of the wild-type enzyme.

CHAPTER III

RESULTS

This project consisted of three primary objectives. The first aim was to construct a wild-type positive control and use that control to determine ideal conditions for a high throughput screen. These conditions would be used to screen the generated mutant libraries for phenotypes that displayed increased alkaline tolerance. These conditions must be harsh enough to significantly decrease the activity of the wild-type cyanide hydratase enzyme, while still being sensitive enough to isolate and identify mutants with improved alkaline tolerance. The second objective of the project was to test a previously utilized error-prone PCR method and confirm its effectiveness as a means of random mutagenesis with the target cyanide hydratase gene. This technique will be necessary to both amplify and introduce random mutations into the *Gloeocercospora sorghi* cyanide hydratase gene in order to create random mutant libraries through the use of *in vivo* cloning. The final step was to use the conditions determined in this project to screen the mutant libraries for any phenotypes that displayed notable increases in alkaline tolerance.

Establishment of Assay and Screening Conditions

In order to determine the ideal screening conditions for the picric acid assay, the *G. sorghi* cyanide hydratase (CHT) gene was cloned into a pBC positive selection vector resulting in strain MB5438 which became the positive control strain for this work. The negative control in both experiments was the pBC vector without the *cht* gene. The wild-type positive and negative control were tested under varying conditions in order to determine conditions that could

effectively, yet selectively, identify mutants with noticeable increases in alkaline tolerance. The results from the testing are described below.

Determination of Screening Conditions by Testing Variable pH Levels and Culture Volumes

The assay initially used to determine the screening conditions tested variable pH levels and cell volumes. The volume of cells used in the assay is significant because the amount of enzyme activity observed is directly proportional to the volume of cell culture used. Therefore, the volume of cultures added is an important variable because of the effect it can play on enzyme activity. The assay showed that for all volumes, the *in vivo* CHT fully degraded the cyanide in all reactions at or below pH 10. At pH 10.5, complete cyanide degradation was retained in all but the lowest culture amounts of 20 μL and 10 μL cell volumes. These volumes demonstrated reduced activity as detected by an orange colorimetric change caused by the addition of picric acid. This colorimetric change indicates a failure of CHT to fully degrade all the cyanide in the well. Finally, at pH 11 the wild type displayed the greatest loss of function. When 50 μL of culture volume was tested, full enzymatic activity was observed. However, with culture volumes of 40, 30, or 20 μL, fluctuating levels of decreased activity were recorded, evidenced by varying hues of orange. Finally, 10 μL of culture displayed a red color, similar to the negative control indicating that there was no observable cyanide degradation. This increase in remaining cyanide indicates a decrease in CHT activity, which can be tied to the decreasing volume of cells used in the reaction. A summary of the assay used to determine the initial screening conditions can be found below (Table 3.1).

	Culture Volume Added				
pH Level	10 µL	20 µL	30 µL	40 µL	50 µL
8.0	Yellow	Yellow	Yellow	Yellow	Yellow
8.5	Yellow	Yellow	Yellow	Yellow	Yellow
9.0	Yellow	Yellow	Yellow	Yellow	Yellow
9.5	Yellow	Yellow	Yellow	Yellow	Yellow
10.0	Yellow	Yellow	Yellow	Yellow	Yellow
10.5	Orange-Red	Orange	Yellow	Yellow	Yellow
11.0	Red	Orange-Red	Orange	Orange	Yellow

Table 3.1 Results from the initial assay used to determine the screening conditions for CHT using the wild type control strain MB5438 grown in culture tube

Generation of Random Mutant Libraries

Error-prone PCR (EP-PCR) was used to introduce random mutations into the *cht* gene sequence in hopes of generating mutants that displayed increased alkaline tolerance. Effective mutagenesis is predicted to generate some fraction of null or partially active clones at physiological conditions. In order to ensure that mutations were being introduced into the gene sequences by the mutagenesis techniques, random screens at cytosolic pH (7.8) were done to test the effect of EP-PCR on the *cht* gene. The results of one of those screens can be viewed below (Table 3.2). Approximately half of the colonies generated by EP-PCR displayed a variation in cyanide degradation ability.

Error Prone PCR Mutagenesis					
Trial 1			**Trial 2**		
	Number	Percentage		Number	Percentage
Yellow	52	55.32	Yellow	13	13.83
Orange	33	35.11	Orange	41	43.62
Red	9	9.57	Red	40	42.55
Total	94		Total	94	
Wild-type					
Trial 1			**Trial 2**		
	Number	Percentage		Number	Percentage
Yellow	94	100.00	Yellow	93	98.94
Orange	0	0.00	Orange	1	1.06
Red	0	0.00	Red	0	0.00
Total	94		Total	94	

Table 3.2 Results from an assay designed to test the effectiveness of EP-PCR as a random mutagenesis agent. A mutant library was constructed and screened at physiological pH (7.8), and the results were compared against an assay of the wild-type CHT also screened at pH 7.8. These reactions were carried out for 10 minutes.

Initial Mutant Screening of CHT Activity at pH 11.0

After successful mutagenesis by EP-PCR was confirmed and the screening conditions for the high throughput picric acid assay were determined, the next step was to screen the generated random mutant libraries that were generated though EP-PCR. The initial set of conditions determined in this project required the use of 25 μL of cell culture, 25 μL of 0.1 M CAPS solution buffered to pH 11.0, and 50 μL of 20 mM cyanide solution buffered to pH 11.0. Under these conditions, no mutants with increased alkaline tolerance were identified, despite screening approximately 2,000 putative mutants.

Subsequent analysis showed that no activity was observed in either the wild-type enzyme (positive control) or in the pBC vector (negative control) under the test conditions. While no cyanide degradation would be expected for the negative control, the wild-type positive was expected to show low levels of activity in order to ensure that the screening conditions did not completely denature the enzyme. A mutant with improved alkaline tolerance can be directly compared with a low activity wild-type enzyme, and this kind of comparison would be a good indicator of a positive target mutant. No low level activity was readily identifiable in the positive control under the initial conditions and several control assays were done to test for any wild-type activity at the determined conditions.

To confirm that the wild-type enzyme retained activity, a picric acid assay was done at pH 8.0, with the same volume of cells, concentration of cyanide and time of reaction. The assay also confirmed that the wild-type control in this assay was capable of fully degrading the cyanide in 30 minutes. The same positive control was also subjected to a mutant screen at pH 11.0 with the

same cell volume, cyanide concentration, and time of reaction. The screen at these conditions produced no measurable activity in the wild-type enzyme after 30 minutes. Finally, to test for any observable activity by the wild-type at the initially determined conditions, a 24 hour reaction was conducted. This test also showed no activity by the positive control, indicating that the originally determined conditions were too stringent and denaturing the enzyme before any observable activity could occur. A summary of these control tests are located below (Table 3.3).

Assay Conditions	**Cyanide Degradation in Wild-type**	**Cyanide Degradation in Negative Control**
pH 8.0/30 min	Full	None
pH 11.0/30 min	None	None
pH 11.0/24 hours	None	None

Table 3.3 Summary of testing initial screening conditions for wild-type activity. At pH 8.0 mutants displayed a range of activity, from full to no activity, which supports the effectiveness of random mutagenesis.

Optimization of Screening Conditions by Testing Variable pH Levels and Time of Reaction

The conditions initially determined for the picric acid screen failed to produce any positive mutants. Therefore a second set of assay conditions was developed to identify a less stringent environment which might prove more sensitive to increased alkaline tolerance. This second assay assessed the effect of different pH levels with varied times of reaction. In this procedure, the wild-type CHT tested in the pH 10 environment showed full cyanide degradation, by producing a yellow color in response to the added picric acid at all time intervals. The wild-type

positive control in the pH 10.5 environment reacted with the picric acid to form an orange color, indicating partial function in the wild-type enzymes. Finally, the wells with the mutants at pH 11 turned red with the addition of picric acid, showing a great loss of function, possibly deactivation. The negative control turned dark red with the addition of picric acid, indicating a complete absence of activity. A summary of the results is presented below (Table 3.4) (Figure 3.1 and 3.2).

pH 10.0			**pH 10.5**			**pH 11.0**		
	Strain			**Strain**			**Strain**	
Time of Reaction	**5438**	**4105**	**Time of Reaction**	**5438**	**4105**	**Time of Reaction**	**5438**	**4105**
10 min	0.299	2.823	**10 min**	0.897	2.980	**10 min**	1.832	3.376
15 min	0.3123	2.744	**15 min**	0.650	3.103	**15 min**	1.737	3.343
20 min	0.273	2.53	**20 min**	0.600	2.859	**20 min**	1.580	3.227
25 min	0.199	2.190	**25 min**	0.455	2.690	**25 min**	1.163	3.040

Table 3.4 Average absorbance at 540 nm indicating the amount of cyanide after reaction. The greater the absorbance, the greater the amount of free cyanide that remained after the time of reaction. The assay compares the effects of time of reaction and pH on the degradation of cyanide by CHT. MB5438 is the wild-type positive control, and MB4105 is the negative control.

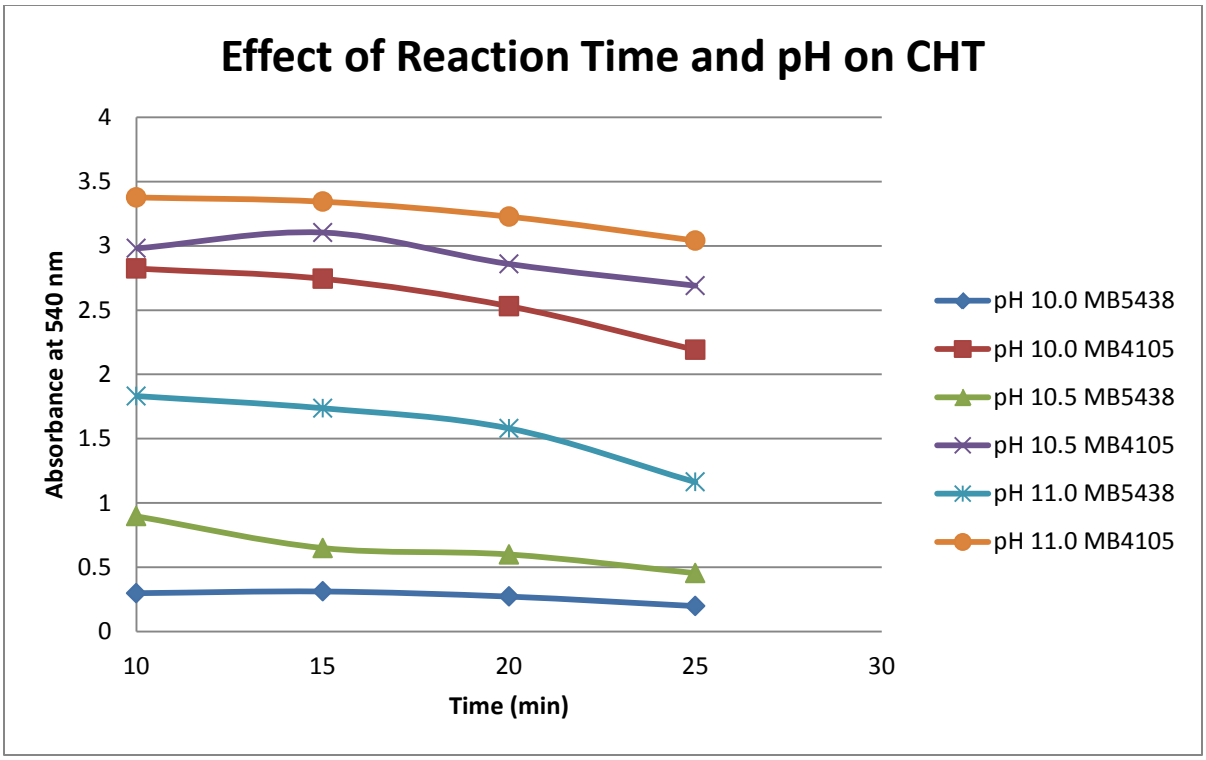

Figure 3.1 Graph of average absorbance values at 540 nm used to demonstrate effect of reaction time and pH on cyanide degradation by CHT. Lower absorbance means less cyanide remaining. MB5438 is the wild-type positive control, and MB4105 is the negative control. Time of reaction was measured from a minimum of 10 minutes to a maximum of 25 minutes. Activity was measured at pH 10.0, 10.5, and 11.0.

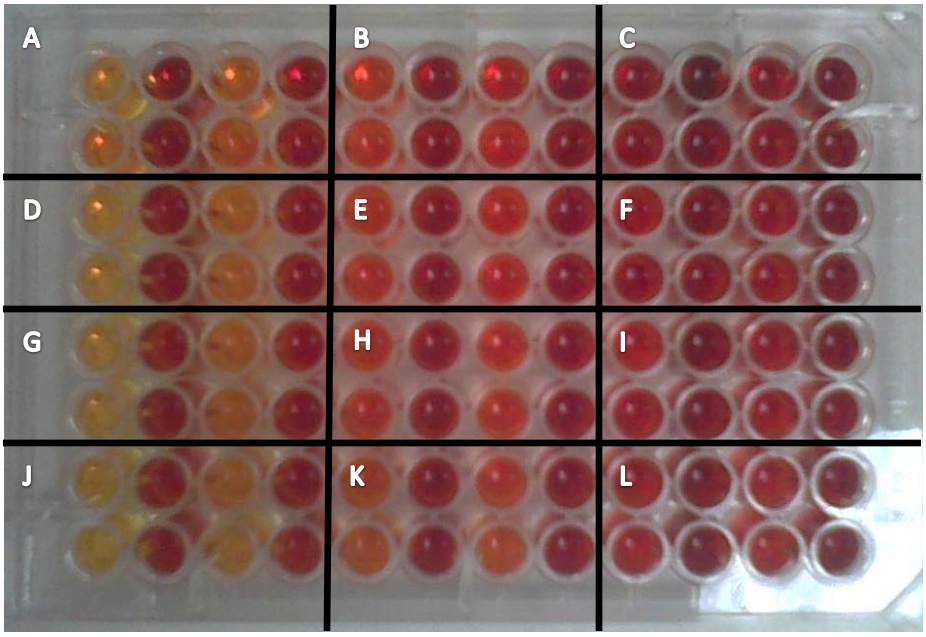

Figure 3.2 Picture of the assay comparing the effect of pH and time of reaction on the degradation of cyanide. Columns alternate with positive wild-type (MB5438) and negative control (MB4105). The following describes the pH and time of reaction for each of the 12 sections: A) pH 10.0/10 min; B) pH 10.5/10 min; C) 11.0/10 min; D) pH 10.0/15 min; E) pH 10.5/15 min; F) pH 11.0/15 min; G) pH 10.0/20 min; H) pH 10.5/20 min; I) 11.0/20 min; J) pH 10.0/25 min; K) pH 10.5/25 min; L) 11.0/25 min;

Optimized Mutant Screening Measuring CHT Activity at pH 10.5

In response to the inefficient screening methods originally determined, experiments were conducted to identify new screening conditions. As previously described, the refined mutant screening method required 50 µL of cells and 50 µL of 20 mM cyanide 0.1 CAPS solution buffered to pH 10.5. Approximately 500 mutants have been screened so far using the newly determined assay conditions. While no positive alkaline tolerant mutants were identified by these conditions, a gradient of activity has been observed. The addition of picric acid reveals

varying shades of orange and red, to indicate the varying levels of cyanide degradation conferred by the random mutations. The wild-type enzyme produces an orange color, demonstrating a decreased activity than normally observed at physiological pH. Screening will continue at these conditions in hopes of isolating a mutant enzyme with increase alkaline tolerance.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The primary objective of this research is to determine picric acid assay conditions that can be used to screen random mutant libraries and also isolate alkaline tolerant mutants of the cyanide hydratase (CHT) found in *Gloeocercospora sorghi*. Mutants isolated by this screening method could be used as a bioremediation agent in the metal mining industry, due to CHT's ability to degrade cyanide. The project required the completion of several objectives before mutant screening could begin. These first of these objectives was to determine the conditions for a high throughput picric acid assay that could be used to screen mutant *cht* genes for phenotypes that display increased alkaline tolerance. The second objective was to adapt a random mutagenesis technique, error-prone PCR (EP-PCR) and test the technique for the production of null mutants, which serve as evidence of mutagenesis. After these two techniques have been developed, mutant library screening can begin.

The first step of this research was to construct a positive control strain (MB5438) to determine screening conditions for a high throughput picric acid assay that will be used to isolate alkaline tolerant mutants. Initially, these conditions were determined empirically by testing cell cultures grown overnight at varying pH levels and culture volumes. The results of this experiment indicated that *in vivo* CHT showed activity in a very alkaline environment (pH 11.0) using a volume of cells (25 μL) in a 100 μL total reaction.

Our lab has previously used EP-PCR as a way of constructing random mutant libraries. EP-PCR utilizes a low fidelity polymerase and the addition of manganese to the PCR reaction in order to increase the error rate of the reaction. EP-PCR typically produces mutations at a frequency of 3 to 5 mutations/kb, which amounts to approximately one to five amino acid changes in the target protein (Wang 2012). Therefore, EP-PCR is not meant to confer massive structural or mechanistic changes that would cause a drastic increase in alkaline tolerance, but instead provide a wide array of slight structural variation. In order to generate mutant libraries, the mutants produced with EP-PCR were cloned into electro-competent bacterial cells using an *in vivo* cloning technique. The *in vivo* cloning technique uses electroporation to stimulate the uptake of both the mutant *cht* genes and the pBC vector while using the lambda recombinase system to combine the two fragments *in vivo*. After the recovered cells were plated, the individual colonies were grown in separate wells in a 96 well plate. Each plate consisted of 94 mutant cells and also contained a positive and negative control in the same plate. Because of this setup, the mutant libraries were screened all at once using the picric acid assay. A typical EP-PCR electroporation generated a library of about 500 or more independent transformants.

To determine the effectiveness of the EP-PCR protocol, tests were run to demonstrate that null mutants could be generated. The picric acid assay was run using mutant libraries at the enzyme's optimal pH (7.8) to determine if the EP-PCR was inducing random mutations that were affecting the enzyme's phenotype. Clones generated by EP-PCR and *in vivo* cloning should show a varying degree of activity due to random mutagenesis; this activity should range between fully activity and a complete loss of degradation. The assays done to check the effects of the EP-PCR have demonstrated this range of activity as displayed in Chapter III (Table 3.2).

After screening approximately 2,000 mutants from the EP-PCR library, no positive mutants were identified. This suggested a possible problem with the initial screening conditions, so they were reviewed. Testing showed that no activity was observed with the positive control under identical conditions. Further evaluation showed that the cell density of the original cultures used in testing was substantially different from the density of the cell cultures being screened. The cell cultures used to determine the initial screening conditions had been grown in overnight culture tubes, whereas the mutant libraries were grown in 96 well plates. Cultures grown overnight in culture tubes have a greater cell density than those grown in 96 well plates and therefore, display greater activity due to the increased number of enzyme-expressing bacteria. Since the mutant libraries were grown in 96 well plates and consequently to this lower cell density, I hypothesized that the initial screening conditions were too stringent, and the wild-type enzyme was inactivated during the assay before activity could be observed. To confirm this hypothesis, a test for cyanide degradation was performed; a 25 µL sample of CHT culture grown in a 96 well plate was inoculated with 25 µL of 0.1 M CAPS buffered to pH 11.0 and 50 µL 20 mM cyanide in 0.1 M CAPS solution buffered to pH 11.0 for 24 hours. The results after even 24 hour of reaction time displayed no degradation of cyanide and indicated that the wild-type enzyme was being inactivated before any cyanide breakdown could occur.

After realizing that the initial conditions were not sensitive enough to detect improved alkaline mutants, new conditions had to be defined. A less stringent assay regimen would allow small increases in activity to be easily noticeable. With this idea in mind, another test was developed in order to optimize the screening process for cells grown in a 96 well plate, as well as for

positive mutant identification. The optimization process focused on the effect of reaction time and pH on cyanide degradation; of these variables, pH appears to be the condition most affecting activity. This assay also showed that the time of the reaction plays a minor role as a condition for cyanide degradation in comparison to the pH of the reaction as see in Chapter III (Figures 3.1 and 3.2). The objective of optimizing the screening assay was to select conditions that could sensitively, yet effectively isolate mutants with notable increases in alkaline tolerance.

The enzymatic activity displayed at pH 10.5 seemed to be ideal for screening the CHT enzyme; while the enzyme failed to completely degrade all the cyanide in the reaction, the wild-type did display partial activity at pH 10.5. Because activity was observed in the wild-type, the assay does not provide conditions so adverse that they will stifle even improved enzymes, and in fact will more likely identify mutants that display a significant increase in activity. Also, the speed of the assay allows for efficient and rapid screening, while still allowing for the identification of only significant alkaline tolerant mutants. Given enough time and repetition, previous results in the lab suggest that this screening condition should allow the isolation of a functional, alkaline tolerant mutant (Abou-Nader 2012, Wang 2012).

With this revised screening regiment in hand, this project is ready to continue with the screening of mutant libraries for alkaline tolerant mutants. When such a mutant is found, the DNA sequence of the *cht* gene will be determined in order to predict the change in the amino acid sequence of the enzyme. There are three kinds of mutations that are predicted to increase apparent pH tolerance. The first kind is a mutation occurring in the promoter of the *cht* gene resulting in increased expression of the wild-type enzyme (Abou-Nader 2012). This results in a false positive due solely to increased amounts of enzyme and is a kind of mutation that our lab is

not interested in. The other two categories of mutations confer a change in the enzyme that results in improved alkaline tolerance. This can be accomplished through mutations that confer either increased structural stability or mutations that alter the mechanism of reaction (Wang 2012). Our lab is very interested in these two types of mutations, as they can provide fundamental insight into the structure and function of the target enzyme, allowing for a more directed approach of enzyme engineering. In addition to mutation categorization, further testing for metal inhibition will also be done to explore the mutant's future as a bioremediation agent used in the mining industry for the remediation of cyanide waste.

This research has successfully generated a protocol for producing mutant libraries of CHT, and a procedure for a high throughput picric acid screen was also determined. One of the most difficult parts of this type of research is the development of an efficient and sensitive screen that can identify target mutants. Now that such a screen has been developed, the future appears promising. The potential mutants that could be isolated with these methods will not only provide the lab with potential bioremediation agents, but with a greater knowledge and understanding of how this enzyme functions and the role of key amino acid residues. This greater understanding of the structural and catalytic effect of individual residues on the efficiency and alkaline tolerance of enzymes could eventually help the in the rational creation of bioremediation agents from these biological molecules. In conclusion, continued research on this enzyme will help to provide creative and environmentally sensitive solutions to the problems caused hazardous mining waste production and storage.

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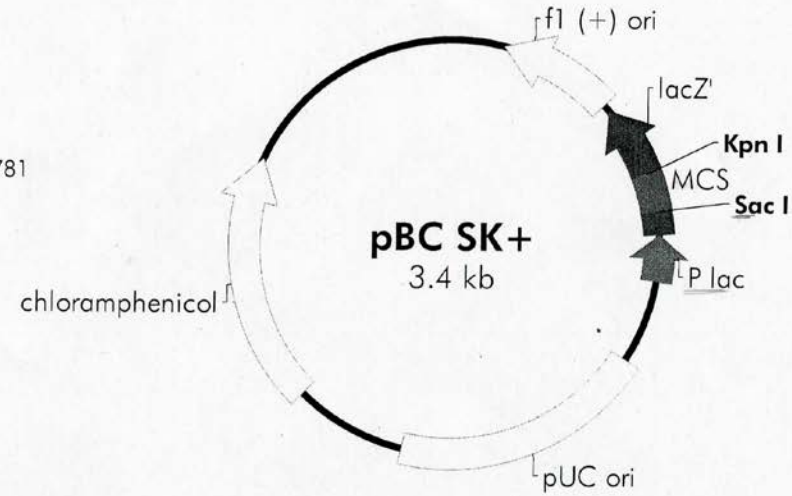
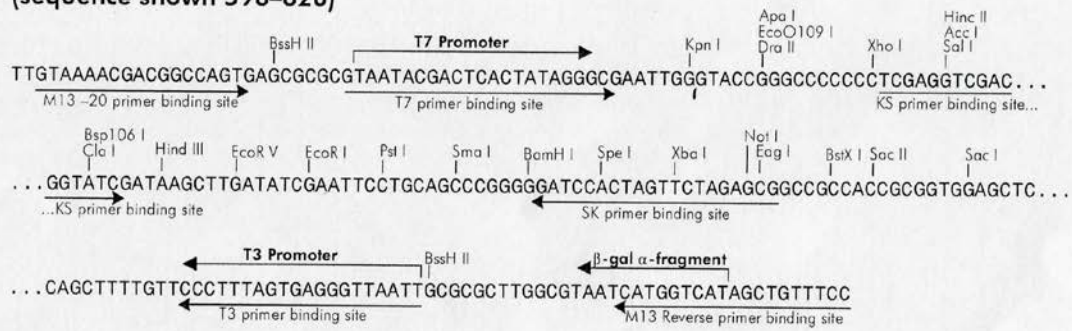
APPENDIX A

PLASMID MAPS

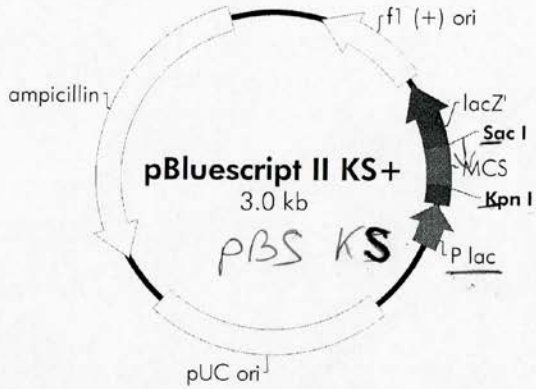
f1 (+) origin 135–441
β-galactosidase α-fragment 460–816
multiple cloning site 653–760
***lac* promoter** 817–938
pUC origin 1158–1825
chloramphenicol resistance ORF 2125–2781



pBC SK (+/–) Multiple Cloning Site Region
(sequence shown 598–826)



f1 (+) origin 135–441
β-galactosidase α-fragment 460–816
multiple cloning site 653–760
***lac* promoter** 817–938
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1976–2833



pBluescript II KS (+/–) Multiple Cloning Site Region (sequence shown 598–826)