GENETIC OPTIMIZATION FOR ALKALINE pH OF A CYANIDE DIHYDRATASE FROM *PSEUDOMONAS STUTZERI*

An Honors Fellows Thesis

by

ALVARO EUGENIO RODRIGUEZ MENDOZA

Submitted to the Honors Program Office
Texas A&M University
in partial fulfillment of the requirements for the designation as

HONORS UNDERGRADUATE RESEARCH FELLOW

April 2011

Major Subject: Microbiology

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Approved by:

Research Advisor:	Michael J. Benedik
Associate Director of the Honors Programs Office:	Dave Louis

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ABSTRACT

Genetic Optimization for Alkaline pH of a Cyanide Dihydratase from
Pseudomonas stutzeri. (April 2011)

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Department of Biology

Even though cyanide is highly toxic, it is commonly found in industrial waste generated by several industries such as metal plating and mining. This chemical is hazardous for many organisms, including mammals, because it inhibits key factors in the respiratory pathway. Surprisingly, several microorganisms can degrade and even survive cyanide's presence. These microbes, fungi and bacteria, count on a variety of cyanide degrading enzymes that break cyanide into less toxic compounds. An example is the cyanide dihydratase ($CynD_{stut}$) found in *Pseudomonas stutzeri AK61.* The enzyme operates optimally around pH 7-8, but most polluted waters have much higher alkaline pH. The aim of this research project is to construct genetic mutants for this enzyme that are able to operate in these highly alkaline environments. The DNA that encodes the enzyme has been cloned into common *Escherichia coli* where it makes functional protein. We show how using methods such as error prone PCR amplification that creates mutations, strains of *E. coli* were screened for cyanide degrading activity at pH 10 and how we identified a

mutant that tolerates these conditions. These novel mutant enzymes can then be analyzed for improved properties useful for bioremediation of cyanide waste waters.

DEDICATION

To my family.

ACKNOWLEDGEMENTS

I would like to thank my project advisor and mentor Dr. Michael J. Benedik for time, help, support, patience, and encouragement through 4 years I was part of his lab. Also I would like to thank Mary Abou-Nader for the encouragement, guidance and support for me to achieve this project.

Special thanks to Dr. Dave Louis, my Honors advisor for the opportunity to carry this research project.

I also would like to thank Benedik's Lab graduate students Allyson Wakefield, Jason Park, and Khrithika Kumar for the help given and for making me feel part of this lab.

Finally, thanks to my mother, father, and sisters, and cat for the support given through these years.

NOMENCLATURE

CynD	Cyanide Dihydratase
$CynD_{pum}$	Cyanide Dihydratase of *Bacillus pumilus*
$CynD_{stut}$	Cyanide Dihydratase of *Pseudomonas stutzeri*
CHT	Cyanide Hydratase
epPCR	Error Prone Polymerase Chain Reaction
PCR	Polymerase Chain Reaction

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Cyanide is commonly found in nature it is used as a source of nitrogen and protection by microorganisms. It is produced by plants, including important crops like corn and cassava, and animals(8). Cyanide is a hazardous chemical for many living organisms, ranging from aquatic life to humans, because it inhibits respiration by tightly binding to cytochrome oxidase (18). Although toxic, cyanide is extensively used in manufacturing synthetic fabrics, gold extraction, herbicide production, and pharmaceuticals, resulting in cyanide containing wastewater. This creates the necessity of degrading it before its release to the environment. To achieve this end many chemical, physical, and biological methods have been developed. The latter offers cost and environmental benefits when compared to the former ones. An example is microbial treatment methods that rely upon a variety of cyanide degrading enzymes found in bacteria, fungi, and plants. Their effectiveness and usefulness for bioremediation of cyanide depends on the different solute contents of wastewaters and their pH, all of which can affect performance.

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This thesis follows the style of the Journal of Bacteriology.

Specific features of enzymes can often be improved on by using directed evolution methods such as error prone PCR in conjunction with high throughput screening methods. This work intends to use these methods to improve on the pH tolerance of a cyanide degrading enzyme (cyanide dihydratase) from *Pseudomonas stutzeri.*

Cyanide degradation methods

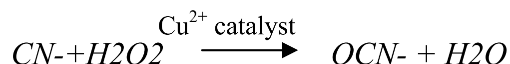
Cyanide is a highly toxic chemical that has been described as having a bitter almond odor and affects the uptake of oxygen by organisms by inhibiting Cytochorome C (18). The term "cyanide" is used to describe any compound containing the CN^- ion and exists in a free form (CN- ion) or in complexed forms depending on pH, the presence of compounds such as metals, or temperature. Another important characteristic of cyanide is its low boiling point (25.7 ºC) which makes it a volatile solute. All these are important when any wastewater remediation method is developed for industrial use (18, 22). Industrial wastewaters contain high cyanide concentrations, which can range from 0.01mg/l to 17-1500 mg/l depending on the industry, in comparison the levels commonly found in unpolluted waters are much lower (0.001-0.05 mg/l). These have to be reduced to acceptable levels, between 5 mg/l to 10 mg/l when released to sewer systems and ultimately to as low as less than 200 µg/l final concentration for potable water (18, 22). To achieve the remediation to the established guidelines several treatment options exist, such as physical methods, chemical, and biological, this last one offering a cost effective and environmentally friendly solution.

Chemical alternatives for cyanide waste treatment comprise ozonation, alkaline chlorination, SO_2/air (INCO process), and the hydrogen peroxide process (2, 15). Those last two have been the most successful chemical methods for degrading cyanide around the globe. They can be summarized by the following equations:

INCO Process

$$CN^- + SO_2 + O_2 + H_2O \longrightarrow OCN^- + SO_4^{2-} + 2H^+$$

Hydrogen Peroxide Process



$$CN^- + H_2O_2 \xrightarrow{Cu^{2+}\ \text{catalyst}} OCN^- + H_2O$$

The SO_2-INCO process mixes sulfur dioxide and air with cyanide at an alkaline pH to efficiently produce sulfuric acid, generating sludge hard to separate from water, a problem partially solved by the addition of nitric acid (23).

Degradation by using hydrogen peroxide (Degussa process) consists of oxidizing cyanide by adding excess hydrogen peroxide and copper as a catalyst to the cyanide containing water producing cyanate. Although it is effective when used for weak acid cyanide metal complexes it is not effective when used to degrade strong acid cyanide-metal complexes (23). Although successful, concerns about these methods exist due to environmental regulations and their cost, specifically for large scale use in the mining industry.

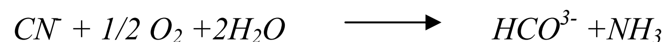
Physical methods to remove cyanide, dilution, dialysis, adsorption, on the other hand have been widely used by the mining industry (23). Traditional physical methods were

primarily used as complementary methods. As technology has improved so have the physical methods. This technology effectively lowered high cyanide concentrations compared to previous treatments (2).

Even though physical technologies have lowered their cost and improved their efficiency over time, biological methods offer feasible and potentially cost effective options. These depend on a variety of degradative metabolic pathways that have evolved for detoxification of cyanide, a substance commonly produced by plants and fungi (2). Their use in the treatment of cyanide containing waters goes back to the early 1900's, although these have not been commercially used until the early 1980's.

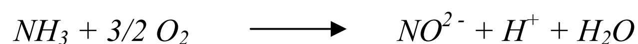
A good example of a successful full-scale application of a biological treatment to degrade cyanide is the Homestake Mining Co.'s process. The process consists of two major steps, a degradative stage and a nitrification stage. The first being the oxidative breakdown of cyanide followed the gathering, absorption and precipitation of free metals into a biofilm. Cyanide is then degraded into carbonate, ammonia, and sulfate. The ammonia is then converted into nitrate in the nitrification step. This is summarized in the following equations:

Degradative stage

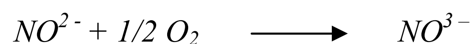


$$CN^- + 1/2\ O_2 + 2H_2O \longrightarrow HCO^{3-} + NH_3$$

Nitrification Stage



$$NH_3 + 3/2\ O_2 \longrightarrow NO^{2-} + H^{+} + H_2O$$



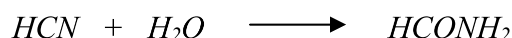
$$NO^{2-} + 1/2\ O_2 \longrightarrow NO^{3-}$$

In this process a microbial consortium is added to the waste directly, and in turn the different organisms perform the conversion to intermediates that are then used by other organisms. This approach has a disadvantage in the time and cost of development needed for these systems(2). Other approaches have used immobilized pure culture lysates of cyanide degrading organisms (3). A third option that has been suggested but has not be thoroughly considered is the use of pure enzyme or enzyme extracts to achieve cyanide degradation (11).

This third option seems interesting as a variety of fungi and bacteria have been found to depend on a single enzyme to breakdown cyanide to less toxic products. These enzymes belong to a branch of the Nitrilase superfamily, the nitrilase branch. Nitrilases depend on a hydrolytic pathway to breakdown nitriles (R-CN) producing different compounds depending on the addition of one or two H_2O molecules(14). In this family two enzymes, cyanide hydratase (CHT) and cyanide dihydratase (CynD) show interesting properties, do not require additional cofactors or substrates to degrade cyanide and therefore could be used for bioremediation (10, 11).

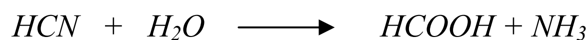
Cyanide degrading enzymes

Cyanide hydratases are found in fungal organisms such as *Fusarium lateritum, Gloeocospora sorghi, and Leptosphaeria maculans.* These homologous enzymes perform the hydrolysis of nitriles to formamide by adding one water molecule.



$$HCN + H_2O \longrightarrow HCONH_2$$

In contrast, cyanide dihydratase (cyanidase) is found in bacteria such as *Bacillus pumilus* and *Pseudomonas stutzeri* AK61. These homologous enzymes hydrolyze nitriles to ammonia and their corresponding carboxylic acid by adding two water molecules.



$$HCN + H_2O \longrightarrow HCOOH + NH_3$$

In the case of both enzymes comparison studies to determine their pH activity profiles, thermostability, metal tolerance, and kinetic constants exist (10). The results for these studies show that CHT was the least thermostable of the cyanide degrading enzymes compared to Cyanide Dihydratases $CynD_{pum}$ and $CynD_{stut}$, but had the highest affinity for KCN. As for the metal tolerance in respect to activity, it depended more on the species from which the enzyme was purified. In the case of all three enzymes, maximum activity was shown between pH 7-8 for both the wildtype enzymes and a hexahistidine tagged version (10, 20).

A significant body of information is available on $CynD_{stut}$ including the determination of its DNA sequence, a proposed structure and a proposed mechanism (10). It has been expressed in *Escherichia coli*, a well studied model organism (21).

The structure $CynD_{stut}$ is described as a spiral structure that consists of 14 subunits with two fold symmetry with 4 surface interactions important for the spiral formation by the subunits. The enzyme has a catalytic triad made up of glutamic acid residue, a lysine and a cysteine residue. The glutamate serving as a base allows the nucleophilic attack by the cysteine to occur on the nitrile. The lysine can then hydrolyze the nitrile that produces ammonia. This is followed by the glutamate activating a second hydrolysis step that produces formic acid (10).

Directed evolution

The range of enzyme catalytic features can prove useful for many applications yet in many occasions fall short and need to be improved. Enzyme engineering allows improvement of their catalytic properties for commercial use. Although a rational approach to engineering them would be the easiest option, the understanding of the relationship between sequence, structure and function is necessary but is not available at the moment for the cyanide degrading nitrilases. Directed evolution, just as Darwinian evolution does, requires generating molecular diversity by introducing random mutations and a screening or selection process that looks for improved enzyme variants(4, 6). This approach is most useful when amino acids important for function have not been

established (5). By introducing a few changes in the sequence, functional changes are generated in enzymes. These functional changes can be driven under a laboratory's controlled conditions to improve specific characteristics. To generate the molecular diversity, error prone PCR mutagenesis and *in vitro* recombination are the most widely used techniques. Once a mutant library is generated, enzyme variants are screened for any improvement, which is an essential step that needs to be fine-tuned for small activity changes.

Mutagenesis can be achieved through chemical mutagenesis, addition of nucleotide analogs, mutator strains, and low fidelity DNA polymerases, this latter being the simplest and most versatile method. Taq polymerase, which lack proofreading activity, in specific has an error rate of 0.001-0.02% nucleotide per pass of the polymerase, the variation explained by different reaction conditions, that can add up to a 2% error rate per PCR reaction. It is this Taq polymerase and reaction conditions that make an effective mutagenesis of a gene during a PCR reaction and is used for epPCR.

Error Prone PCR does have its limitations. One limitation is an amplification bias due to PCR's exponential amplification that can cause an early mutation to be overrepresented in a single reaction. This can be easily resolved by combining several epPCR reactions reducing that overrepresentation of a mutation in the library. A second problem is that epPCR using Taq polymerase can presents a strong bias towards transitions of nucleotides. This can be solved by modifying reaction condition by adjusting nucleotide levels. A third limitation that affects the size of the library that can be produced by using

epPCR is the recombination step between an insert and a vector. The *in vitro* recombination method using ligase is commonly used to achieve the recombination between the insert and the vector. Its effectiveness depends on the ligation rate affecting the size (17). The recombination step can be improved by using *in vivo* recombination which has been shown to significantly increase the library size, which is extremely important as it increases the chances to find a mutant with the desired improvement (1). *In vivo* recombination depends on bacteriophage λ recombination genes which offer a way to transform linear DNA into *E. coli*. This is done by taking advantage of the inhibition of the *E.coli's* RecBCD complex, which normally degrades linear DNA by Gam, and the Red recombination system made up by Exo that has 5'-3'exonuclease activity and Bet that promotes *in vivo* recombination of the co-transformed vector and the insert. These genes are present in a vector hat allows their expression (13).This method has been further improved by the addition of a plasmid with the F' plasmid's *ccdB* toxin gene that allows positive selection for recombinant molecules. The selection is achieved when recombination is successful due to the replacement of the *ccdB* gene by the insert (1).

CHAPTER II

GENERATION OF RANDOM MUTANT LIBRARIES AND SCREENING PROCESS

Introduction

Two main pathways can be followed when engineering enzymes: rational design or directed evolution. The main downside of the first is that knowledge of structure and function is still fragmented, making the latter look more appealing. Directed evolution is "*in vitro* natural evolution" consisting of methods that generate diversity and screening and selection methods to permit identification of improved enzyme features such as thermostability, activity in artificial environments, and substrate specificity (12). For directed evolution to be successful enzyme expression in a microbial host, a random diversity generation method and a selection (screen) method for the desired feature are allrequired. All of which have been met in the case of *Pseudomonas stutzeri's* cyanide dihydratase in this work.

In this chapter, I describe the strategy used to generate a mutant version of the *P. stutzeri* CynD that maintains activity at high pH, thereby improving $CynD_{stut}$'s potential for bioremediation. This will be done by generating a diverse library of mutants using epPCR followed by *in vivo* cloning into *E. coli* using standard and generating clones, which will then express the randomly mutated copies of the gene. Thousands of individual clones will then

be grown in wells of a microplate and can be assayed to look for activity at alkaline pH using a variation of the picric acid-based method (Fig.1) that measures changes in cyanide concentrations (9).

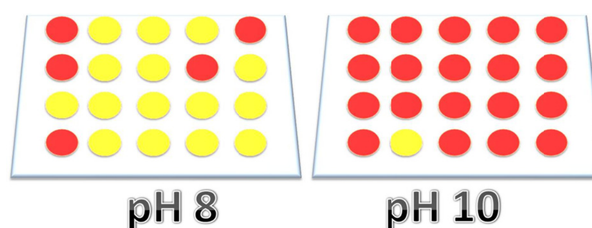


Fig. 1 Picric acid based method. The yellow color indicates high cyanide dihydratase enzyme activity, while the red no activity.

Materials and methods

Culture media and reagents

The *E.coli* strains used were grown in LB broth and when required ampicillin or chloramphenicol was added to concentrations of 100 µg/ml and 25 µg/ml, respectively.

Taq 2 x Master Mix and restriction enzymes where purchased from New England Biolabs (Ipswich, MA). Bio-X-Act short mix was bought from Bioline (Boston, MA).

TABLE 1. *E.coli* strain and plasmid description

Strains and plasmids	Description	Reference
pBS (KS+)	Amp^r *E.coli* cloning vector	Agilent
pBC (SK+)	Cam^r *E.coli* cloning vector	Agilent
MB4091	Strain carrying DH10B pKD46 with *red* recombination genes	Lab stock
MB3577	Strain carrying pBS (KS+) carrying $CynD_{stut}$	Lab stock
MB3817	Strain carrying pBS (KS+) carrying mutant $CynD_{pum}$	Lab stock
MB4108	Strain carrying pBC(SK+) carrying $CynD_{stut}$	This work
MB4712	Strain carrying pBC (SK+) carrying mutant $CynD_{pum}$	This work
MB4955	Strain carrying pBC (SK+) carrying mutant $CynD_{stut}$	This work
MB4105	Strain carrying pBC (SK+) with *ccdB* toxin	Lab stock

Bacterial strains and plasmids

MB4091 strain is DH10B pKD46 carrying red recombination genes and was the recombination host used. Plasmid p4105 is pBC (SK+) with ccdB toxin (1) gene was the vector for was used for *in vivo* recombination. Plasmid p3577 is pBS SK+ carrying $CynD_{stut}$ as XbaI-XhoI fragment that was used as PCR template. MB4108 strain is

carrying pBS(KS+) carrying $CynD_{stut}$ and MB4712 pBs (KS+) carrying mutant $CynD_{pum}$. For a complete list see Table 1.

Error-prone PCR (epPCR)

The full length cyanide dihydratase gene from p3577 was used for the error-prone PCR. The primers -60M13F (5'-GCG AAA GGG GGA TGT GCT GCA AGG) and -60M13R (5'- CAC TTT ATG CTT CCG GCT CGT ATG) are outside the cloning sites and were used to amplify the gene. A 50µl total volume epPCR reaction was performed using 25 µl of Taq 2x master mix (New England Biolabs) , 100 ng of each primer, 1 µl of 10 mM$MnCl_2$ stock and 21 µl MqH_2O. The reaction conditions were the following: 1 cycle at 95ºC for 2 min, 25 cycles of 30 sec at 95ºC, 55ºC for 1 min, 72 ºC for 1 min, and 1 cycle for 7min at 72ºC. The reaction was ethanol precipitated and resuspended in 20 µl of Tris EDTA buffer. DNA concentration was determined using a nanodrop spectrophotometer by measuring the A_{260} . For the PCR reaction 25ml of Bio-X-Act Short mix (Bioline), 100 ng of each primer, and 23 µl MqH_2O where used. The reaction conditions were the same as for the epPCR reaction.

Positive selection vector preparation

The vector used was p4105 (1). The vector was digested using XbaI and XhoI for 3 hrs at 37ºC.The DNA was ethanol precipitated, resuspended in Tris EDTA buffer and concentration was determined by measuring A_{260}, and stored at -20ºC.

Transformation

MB4091 was used as recipient strain for *in vivo* recombination as it carries the temperature sensitive plasmid pKD46 carrying the λ Red and Gam genes expressed by inducing the pBAD promoter with arabinose at 30ºC(7). MB4091 cells were grown in LB broth to an 0.3 O.D(600nm) and induced with 0.1% arabinose for 1 hr at 30ºC.The epPCR product and positive selection vector added to a ratio of 0.25/1 pmole ratio were co-transformed using a Bio-Rad Micropulser into electrocompetent cells, allowed to recover for 30 min at 37 ºC for 30 min, spread on plates with 25 μg/ml chloramphenicol, and allowed to grow overnight at 37C.

Alkali-tolerant mutant screen

A visual picric acid-cyanide assay was used to detect cyanide degrading activity at pH8 and pH10. Recombinant colonies were manually picked and used to inoculate 170 μl of LB broth with chloramphenicol in 96 well plates and allowed to grow overnight at 37ºC. Tris-base buffer from US Biologicals (Swampscott,MA) for pH 8 and CAPS buffer from VRW(Radnor, PA) at pH 10 were chosen because of their respective useful buffering ranges. 60 μl of 0.1 M Tris buffer (pH 8) or 0.1M CAPS buffer (pH10) with 6.7mM

KCN and 40 µl of each mutant strain culture were added into a 96 well plate. The reaction was allowed to run for 1 hr. of 100µl a solution of 0.5M Sodium Carbonate from EMD Chemicals (Gibbstown, NJ) and 0.6% w/v picric acid from VRW (Radnor, PA) was added to each well to terminate the reaction. The plates were then incubated at 65 ºC for 15 min to develop the reaction and allow the color change. Active clones where identified by a yellow color. Clones active at pH 10 were then picked into LB agar chloramphenicol plates for a second confirmatory screen.

Second screen

Candidate clones were picked and grown overnight in 1 ml LB at 37ºC for a second screen: 250µl of culture and 250µl of 0.1 M CAPS buffer at pH 10 were mixed, the reaction is allowed to proceed for 60 min in 1.5 ml microfuge tubes with KCN added to a final concentration of 4mM. As a control a second reaction at pH 8 using Tris as the buffer was used as a control and terminated after 60 min. The reactions were terminated with equal volumes of the sodium carbonate and picric acid solution.

Sequencing

Interesting plasmid candidates were sequenced as follows: 2.5 µl using BigDye from Applied Biosystems (Carlsbad, California) , 200ng of primer, 2µl template, and 3.5 µl MqH2O were mixed and the reaction conditions were reaction: 34 cycles of 95ºC for 10 sec, 55ºC for 5 sec, and 60ºC for 4 min. The reaction was ethanol precipitated and stored at -20 ºC until the sequences could be determined in the Department of Biology Laboratory for Gene Technologies.

Results

A picric acid assay at pH8 was conducted to do a comparison between the clones from epPCR and a PCR reactions based on the assumption that different phenotypes should be in observed in epPCR clones due to the effects of mutagenesis, but not the control reaction clones. As expected an increase in $CynD_{stut}$ mutants, both nonfunctional and partially functional phenotypes were observed when compared to a normal PCR amplification reaction where few mutants were observed. The results are shown in Table 2.

TABLE 2. epPCR and PCR $CynD_{stut}$ phenotype comparison.

	epPCR reaction Nonfunctional clones/partially functional clones*	Control PCR reaction Nonfunctional clones/partially functional clones*
Trial1	55/80 $^{+}$	3/48^{+}
Trial 2	9/32 $^{+}$	0/32^{+}

*Cyanide degrading activity was tested at pH 8.
$^{+}$ Nonfuctional/Partially functional clones divided by total sampled clones

CAPS buffer effects on phenotype

In previous studies (19) Tris base buffer was used in the picric acid assay for both pH 8 and pH10. Due to Tris base buffer having a useful pH buffering range between pH7 and pH9, it was decided to run the assay at pH 10 using CAPS which has a useful pH range between pH 10 and pH11. To whether CAPs buffer has any effect on activity of $CynD_{stut}$,a comparison assay was done. The assay demonstrated that CAPS was not inhibitory to enzyme activity (Table 3) as similar results were obtained with the different buffers. The main advantage is that pH is better maintained at pH 10.

TABLE 3. CAPS buffer effect on $CynD_{stut}$ activity

Buffer	Tris-base pH 8	Tris-base pH 10	CAPS pH 8	CAPS pH10
MB 3577 *	Y	O	Y	O
MB 3817+	Y	Y	Y	Y
MB3957^	R	R	R	R

*wiltype $CynD_{stut}$ enzyme (positive control)
+alkaline pH tolerant mutant of $CynD_{pum}$ (positive alkaline pH activity control)
^control strain with no CynD (negative control)
Y stands for yellow (High cyanide degrading activity) reaction color , O for orange color (Some activity) and R for red color (low cyanide degrading activity).

Screening of epPCR clones

The wildtype and the $CynD_{pum}$ mutant genes were *in vivo* cloned into MB4091 and transferred from pBS KS+ to pBC-ccdB to allow the use of these as positive and negative controls and to permit direct comparison with the clones generated via the epPCR and *in vivo* recombination methods. The picric acid assay was performed and candidate phenotypes where selected. To detect any false positive candidate clones a second screen was performed. In Table 4 it is possible to see how this second screen allowed distinguishing between false positive candidate phenotypes and possible phenotypes possessing the ability to tolerate alkaline pHs.

TABLE 4. Second screen of candidate phenotypes

Time (min)	LB	MB4108*	MB4712+	MB4955°	J9ˣ
60 (pH10)	R	O	Y	Y	R
60 (pH 8)	R	Y	Y	Y	Y

*wiltype $CynD_{stut}$ enzyme (positive control)
+alkaline pH tolerant mutant of $CynD_{pum}$ (positive alkaline pH activity control)
^LB with no CynD containing strain (negative control)
°Interesting clone
ˣFalse positive clone
Y stands for yellow reaction color(High cyanide degrading activity), O for orange color (Some activity) and R for red color(low cyanide degrading activity).

Sequencing and mutations

After screening about 16,000 of clones generated by epPCR , an interesting clone which showed activity at pH 10 was identified and sequenced. After sequencing several mutations were observed. These mutations included silent mutations, T413C and A656G, and three amino acid changes, E225V, E249D, and N319S (Fig 2). This shows that the process followed in the search for improved tolerance at alkaline pHs was successful by showing that the epPCR generated diversity, *in vivo* recombination allowed producing the clone library and the picric acid screen allowed identifying interesting mutant plasmids with appropriate phenotypes of interest.

```
MAHYPKFKAAAVQAAPVYLNLDATVEKSVKLIEEAASNGAKLVAFPEAFI 50     MB4108
MAHYPKFKAAAVQAAPVYLNLDATVEKSVKLIEEAASNGAKLVAFPEAFI 50     CynDstut
**************************************************        MB4955
                                                          CynDstut

PGYPWFAFLGHPEYTRRFYHTLYLNAVEIPSEAVQKISAAARKNKIYVCI 100    MB4108
PGYPWFAFLGHPEYTRRFYHTLYLNAVEIPSEAVQKISAAARKNKIYVCI 100    MB4955
**************************************************

SCSEKDGGSLYLAQLWFNPEGDLIGKHRKMRVSVAERLCWGDGNGSMMPV 150    MB4108
SCSEKDGGSLYLAQLWFNPEGDLIGKHRKMRVSVAERLCWGDGNGSMMPV 150    MB4955
**************************************************

FETEIGNLGGLMCWEHNVPLDIAAMNSQNEQVHVAAWPGFFDDETASSHY 200    MB4108
                                                          MB4955
FETEIGNLGGLMCWEHNVPLDIAAMNSQNEQVHVAAWPGFFDDETASSHY 200
**************************************************

AICNQAFVLMTSSIYSEEMKDMLCETQEERDYFNTFKSGHTRIYGPDGEP 250    MB4108
AICNQAFVLMTSSIYSEEMKDMLCVTQEERDYFNTFKSGHTRIYGPDGDP 250    MB4955
************************ ***********************:*

ISDLVPAETEGIAYAEIDIEKIIDFKYYIDPVGHYSNQSLSMNFNQSPNP 300    MB4108
ISDLVPAETEGIAYAEIDIEKIIDFKYYIDPVGHYSNQSLSMNFNQSPNP 300    MB4955
**************************************************

VVRKIGERDSTVFTYDDLNLSVSDEEPVVRSLRK 334                    MB4108
VVRKIGERDSTVFTYDDLSLSVSDEEPVVRSLRK 334                    MB4955
******************.***************
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Fig. 2 ClustalW alignment of wildtype and a CynD_{stut} mutant (MB4955) (16). The amino acid changes in the protein are shown in bold

Discussion

The main objective of this project was to use directed evolution to find optimized $CynD_{stut}$ mutants tolerant to alkaline pH. As any directed evolution experiment requires a random mutagenesis method, epPCR, and a screening method for the desired property, that is at pH 10, were used. I was successful in optimizing the methodology and isolating the desired mutant.

I screened for activity using the pH8 as a simple way to test how effective the epPCR method was in producing mutant phenotypes and to optimize my conditions. As shown by the percentage range of mutant phenotypest 28%-63% of the epPCR clones, much higher than the control PCR reaction of the $cynD_{stut}$ gene, my epPCR protocol was effective. This suggests that mutations were introduced during the replication process, which generated a diverse population of molecules, an essential step for directed evolution of the enzyme to be successful. It also demonstrates how epPCR is a practical method for introducing random mutations.

In conjunction with the random mutagenesis step, it was important to develop a useful screen to identify the desired phenotype of the enzyme, in this case activity at alkaline pH. The picric acid assay was chosen as a simple visual screen that detects the relative amount of cyanide, whose degradation depends on the activity of the enzyme. More importantly it allows using different pHs, in this case pH 8 and pH 10, to distinguish between phenotypes, wild type, and possible mutant phenotypes active at pH 10. For this

study, it was decided that the Tris base buffer might not be the best option to maintain a pH at 10 due to its lack of buffering capacity at pH 10. A better buffer, CAPS, was chosen and tested to identify any problems that it could cause for activity of the $CynD_{stut}$ enzyme. The results showed no difference in activity between the wild type enzymes, the alkali-tolerant mutant from *Bacillus pumilus* and a negative control strain with without cyanide dihydratase. Since no difference in activity was observed, it was possible to use CAPS buffer to screen at pH 10 to perform the picric acid screen.

My screening identified two interesting plasmids. The plasmid p4955 showed interesting activity in the second round assay at pH 10 and J9 showing activity in similar to the control wildtype. I chose to only pursue the p4955 mutant whose activity at pH 10 was equal to the control mutant $CynD_{pum}$ which had been previously identified as a mutant cyanide dihydratase mutant tolerant to alkaline pHs.

The sequence of the p4955 showed there were 3 amino acid differences. In Figure 2, a sequence alignment of the wildtype enzyme and mutant MB4955 show the mutations introduced by the epPCR.

In summary the project was successful in that a complex mutant with the desired high pH activity phenotype was identified by the methods presented.

CHAPTER III

SUMMARY AND CONCLUSION

Cyanide is a toxic chemical as it prevents humans, and many other organisms, from using oxygen, yet it is extensively used in many industrial processes. Although cyanide can be found in nature at low levels, it is also found in soil and waters at high concentrations due to its industrial use. This makes cyanide presence an important environmental concern.

The gold mining industry represents one of the biggest cyanide consumers. The reason is that cyanide is essential for one of the cheapest gold extraction processes. The process consists of grinding the ore, adding cyanide that forms a complex with gold dissolving it, raising the pH to avoid cyanide volatizing, recovering the gold, and storing the waste waters. In some situations the cyanide is recovered and used again, but many times it has to be safely removed before it can be released into the environment.

Removing cyanide by chemical and physical methods can be expensive; therefore cheaper biological methods are a good option. These rely on fungi and bacterial enzymes that can break cyanide into less harmful substances. For the gold mining case, Cyanide dihydratases are good candidates for bioremediation as they degrade cyanide into an acid and ammonia, less hazardous substances. Also they have no need for other cofactors and

remain active in the presence of other metals. A problem still remains as these loose activities in alkaline pHs, an important step in the gold extraction process.

Two methods, rational design and directed evolution, exist that allow to improve the features of an enzyme to overcome the pH problem. As rational design of enzymes is still not a viable as many gaps in understanding their function and structure exist, directed evolution offers a proven method that achieves improvements in them. Directed evolution requires a method to generate diversity and a method to select or screen that diversity. Our objective was to use epPCR, *in vivo* recombination and a picric acid assay to generate a library of mutant clones of $CynD_{stut}$ and screen for mutants that tolerate alkaline conditions.

In this study we tested the efficiency of the epPCR method to generate mutant phenotypes including nonfunctional and partially functional phenotypes, the effects on activity CAPS buffer, and the screening methodology to follow to detect interesting phenotypes with the property we were looking for.

We found that epPCR and *in vivo* recombination offer an efficient method to generate diversity phenotypes as these greatly increase (22.5%-63%) when compared to a PCR reaction (0%-6.3%). Also we determined that CAPS has no effect in $CynD_{stut}$ activity, when tested with the picric assay method at pH 8 and pH10, as the results where similar as previously used buffer Tris base. Finally we showed how a second round of screening allows discarding or detecting candidates from the first round screen and demonstrated

by sequencing of one of these candidates that several mutations were introduced by the epPCR method.

Still further characterization of these mutants is needed. The characterization of these includes producing thermostability profiles, a pH profile and metal tolerance profile to allow comparison to the wildtype enzyme. Also knowledge about the structure can be gained by reverting one mutation at a time to the original amino acid and describing the effect of it. It is this characterization process that will allow producing a CynD enzyme that is useful for bioremediation. Furthermore this will improve the understanding of the role of single amino acids that in a future will allow rational design.

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