

# Biodegradation of cyanide via recombinant cyanide dehydratase from *Bacillus pumilus* expressed heterologously in *Escherichia coli*

## Biodegradación de cianuro por la enzima cianuro dihidratasa de *Bacillus pumilus* expresada heterológicamente en *Escherichia coli*

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### ABSTRACT

Despite its high toxicity, cyanide is used in several industrial processes, and as a result, large volumes of cyanide wastewater need to be treated prior to discharge. Enzymatic degradation of industrial cyanide wastewater by cyanide dihydratase, which is capable of converting cyanide to ammonia and formate, is an attractive alternative to conventional chemical methods of cyanide decontamination. However, the main impediment to the use of this enzyme for the biodegradation of cyanide is its intolerance to the alkaline pH at which cyanide waste is kept and its low thermoresistance. In the present study, the catalytic properties of whole *Escherichia coli* cells overexpressing a cyanide dihydratase gene from *Bacillus pumilus* were compared to those of the purified enzyme under conditions similar to those found in industrial cyanide wastewater. In addition, the capacity of whole cells to degrade free cyanide in contaminated wastewater resulting from the gold mining process was also determined. The characteristics of intracellular enzyme, relative to purified enzyme, included increased thermostability (>60% activity at 50°C), as well as greater tolerance to heavy metals, and to a lesser extent pH (20% activity remaining at pH 9.0). On the other hand, enzymatic degradation of 70% of free cyanide (initial concentration 528 mM) in the industrial sample was achieved only after dilution. Nevertheless, the increased thermostability observed for intracellular CynD suggest that whole cells of *E. coli* overexpressing CynD are suited for process that operate at elevated temperatures (50°C), a limitation observed for the purified enzyme.

**Key words:** Biodegradation, cyanide, cyanide dihydratase, gold mining effluents, whole cells

### RESUMEN

A pesar de su alta toxicidad, el cianuro es usado en diversos procesos industriales, y como resultado, grandes volúmenes de aguas residuales de cianuro deben ser tratados antes de su descarga. Una alternativa atractiva a los métodos químicos convencionales de descontaminación es la degradación enzimática por la enzima cianuro dihidratasa, la cual es capaz de convertir cianuro en amonio y ácido fórmico. No obstante, la inactivación de esta enzima a pH superior a 8.5 y su poca termoestabilidad han sido el principal impedimento para la implementación exitosa de esta alternativa de biorremediación. En el presente estudio, las propiedades catalíticas de células completas de *Escherichia coli* que sobre expresan el gen de cianuro dihidratasa de *Bacillus pumilus* se estudian

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bajo condiciones similares a las encontradas en aguas residuales industriales de cianuro y los resultados se discuten en comparación con las de la enzima purificada. Además, se determinó la capacidad de las células completas para degradar el cianuro libre en aguas residuales resultantes del proceso de extracción de oro. Las características de la enzima intracelular, relativa a la enzima purificada, incluyeron un incremento en la termoestabilidad (>60% actividad a 50°C), así como mayor tolerancia a metales pesados y en menor medida al pH (20% actividad residual a pH 9.0). Por otra parte, la degradación enzimática del 70% del cianuro libre en la muestra industrial (concentración inicial 528 mM) se logró solo después de la dilución de la muestra. Sin embargo, el incremento en la termoestabilidad observado para CynD intracelular sugiere que las células completas de *E. coli* que sobre expresan CynD son adecuadas para procesos que operan a temperaturas elevadas (50°C), una limitación observada para la enzima purificada.

**Palabras clave:** Biodegradación, cianuro, cianuro dihidratasa, efluentes de la minería de oro, células completas.

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## INTRODUCTION

Cyanide is highly toxic to most living organisms. The toxic dose in humans is 1 mg/kg body weight, with higher doses potentially causing death within minutes (Vogel *et al.*, 1981). Despite its toxicity, cyanide is widely used in several industrial processes, including the chemical synthesis of polymers, electroplating, and gold mining (Cummings, 2004); with the latter industrial activity posing the largest environmental hazard (Johnson, 2015). Gold mining practices generate large volumes of cyanide-laden waste effluents, usually kept in dams, which need to be properly contained and remediated. Unfortunately, there are numerous examples of the catastrophic effect of accidental spills from mine waste dams (Henderson, 1995; Rico *et al.*, 2008).

In recent years, global gold price has increased 360 % (Swenson *et al.*, 2011). As a result, gold mining has dramatically increased in developing countries. Gold mining in Latin America has grown rapidly, and by 2010 the region contributed 19% of the total world production (Swenson *et al.*, 2011). Unfortunately, poor control and management of the chemicals used in the process have resulted in spills of cyanide, mercury, and other heavy metals into rivers and soil, causing severe injury to exposed human inhabitants, wildlife, and overall biodiversity (Tarras-Wahlberg *et al.*, 2001). A rapid, cost-effective, environmentally-friendly solution for the treatment of the cyanide wastewater generated by gold mining is urgently needed.

Conventional chemical methods used to treat cyanide wastewater have drawbacks, such as high costs, the need for special infrastructure, and the use of chemical reagents that are themselves environmental hazards (Akci, 2003). Considering these limitations, biological methods for cyanide degradation represent attractive alternatives (Dash *et al.*, 2009; Gupta *et al.*, 2010). Specifically, the enzymatic degradation of industrial cyanide wastewater by cyanide dihydratase (CynD) a member of the nitrilase superfamily, has been reported as a plausible alternative (Martínková *et al.*, 2015; Park *et al.*, 2017; Thuku *et al.*, 2009). The appeal of using CynD enzymes

derives from their ability to rapidly convert cyanide into ammonia and formate without any added cofactors.

Jandhyala *et al.* (2003), expressed CynD from *Bacillus pumilus* in recombinant *E. coli* and purified the enzyme using a histidine tag. The recombinant enzyme displayed maximum activity between 37 °C and 42 °C, was inactive at a pH >8.5, and was greatly inhibited by Hg<sup>2+</sup> (Jandhyala *et al.*, 2005). More recently, protein-engineering efforts resulted in CynD mutants and chimeras with increased activity at an alkaline pH and increased thermal stability (Crum *et al.*, 2015; Crum *et al.*, 2016). More recently, a CynD from *Flavobacterium indicum* was immobilized on agar to develop a potentiometric biosensor for detection of cyanide (Kumar *et al.*, 2018). In spite of the success in improving the catalytic properties of purified CynD enzymes, an even more robust catalyst is required for the bioremediation of gold mining cyanide wastewater.

The use of whole cells engineered to overexpress enzymes of interest for industrial processes is appealing because cellular membranes and the cytoplasm can provide some level of protection to intracellular enzymes against detrimental conditions, such as extreme pH, temperature, and inhibitors; thereby making whole cells more suitable for industrial processes (De Carvalho, 2016; Lin & Tao, 2017; Wachtmeister & Rother, 2016). Based on this assumption, and findings by Vargas-Serna and Panay (2019), we hypothesized that whole *E. coli* cells overexpressing CynD could be better suited for degrading cyanide under the harsh conditions present in the cyanide wastewater generated by gold mining.

## MATERIALS AND METHODS

### Bacterial strains and plasmids

*Escherichia coli* DH5 $\alpha$  was used for plasmid DNA replication and *Escherichia coli* BL21(DE3) was utilized for protein expression. The *Bacillus pumilus* C1 cyanide dihydratase gene sequence (GenBank AF492815) (Jandhyala *et al.*, 2003) with an additional C-terminal hexahistidyl tag was codon optimized for *E. coli* protein

expression (GenBank MH917689), synthesized and cloned in a vector PD451 by DNA 2.0 (Atum, Newark, CA, USA). This plasmid, named PD451-BpumCynD, is available upon request.

### Whole cells expressing CynD (*E. coli-CynD*)

Single colonies of *E. coli* BL21(DE3) transformed with the plasmid PD451-BpumCynD were grown in LB media for 16 h at 37 °C and 300 rpm. Fresh cultures were initiated by adding a 100-fold dilution of the overnight culture to fresh LB media supplemented with kanamycin. When the OD<sub>600nm</sub> reached 0.5, the cultures were induced with 0.4 mM IPTG. CynD expression was monitored by SDS-PAGE. Cells were harvested 4 h after induction by centrifugation for 30 min at 20 °C and 5800 rpm using an Eppendorf centrifuge 5804R. Cell pellets were immediately used or stored at -80 °C. Untransformed *E. coli* BL21(DE3) was grown following a similar procedure but without the addition of antibiotics or IPTG. This culture served as a negative control for cyanide degradation.

### Cyanide determination

Cyanide concentrations were measured by the picric acid method (Fisher & Brown, 1952) mixing 500 µL of cyanide-containing solution and 500 µL of 0.5 M sodium carbonate and 1% picric acid. This solution was heated at 100 °C for 5 min and the reaction stopped with 3.5 mL of water. Cyanide concentration was determined measuring absorbance at 520 nm utilizing a standard curve ranging between 0 to 4.0 mM cyanide. For samples with concentration > 4.0 mM the assay supernatant was diluted so the absorbance fell within the linear range of the standard curve.

### *E. coli-CynD* activity measurements

For whole cell kinetics, reactions were carried out in a volume of 1 mL, containing 0.1 mg of cells in 20 mM tris (pH 8.0), and varying amounts of cyanide. Initial velocity rates were calculated from the linear portion of plots of cyanide concentration vs time. Kinetic parameters were determined from plots of initial velocities against cyanide concentration fitted to the Michaelis Menten equation. For total cyanide degradation, pellets of *E. coli-CynD* were brought to an OD<sub>600nm</sub> of 1.1 in: LB broth for growing cells, 20 mM buffer tris (pH 8.0) for resting cells, and 20 mM tris (pH 8.0) with 5% toluene and 1 mM EDTA for permeabilized cells. Total cyanide degradation was determined after 10 minutes of reaction.

Negative controls included untransformed *E. coli* BL21 (DE3) and a solution without the addition of cells which was used to account for cyanide loss through evaporation. All of the results represent the average of three

independent measurements. Percent cyanide removed was calculated using Equation 1, where [Cyanide<sub>0</sub>]: is the initial cyanide and [Cyanide<sub>t</sub>] is the final cyanide concentration.

$$\text{Equation 1: \%Cyanide removed: } \frac{[\text{Cyanide}_0] - [\text{Cyanide}_t]}{[\text{Cyanide}_0]} * 100$$

### Activity at basic pH

For total cyanide degradation at alkaline pH, *E. coli-CynD* cells were resuspended in 20 mM buffer tris (pH 8.0) and the suspension was adjusted to an OD<sub>600nm</sub> of 1.1. Reactions were carried out in either 20 mM tris pH 8.0, 50 mM glycine pH 9.0 or pH 10.0 in a total volume of 1 mL with 5 mM cyanide and 100 µL of the cell suspension. The activity is reported relative to that at pH 8.0. Total cyanide degradation at each pH was determined at 10 min intervals up to 1 hour.

### Optimum Temperature and thermostability

The activity of 0.1 mg of *E. coli-CynD* was determined in a range of temperatures from 35 °C to 60 °C with five-degree intervals. Assay solutions contained 5 mM cyanide in a total volume of 1 mL, 20 mM Tris (pH 8.0). Activity is reported relative to that at 35 °C. All assays were performed in triplicate.

For thermal stability, *E. coli-CynD* was adjusted to an OD<sub>600nm</sub> of 1.1 in 20 mM buffer tris (pH 8.0). The cells were incubated at 42 °C for 75 h. Every five hours the enzymatic activity was determined using 100 µL of the cells. All assays were performed in triplicate.

### Heavy metal inhibition

Cyanide removal was assayed in the presence of FeCl<sub>3</sub>, HgCl<sub>2</sub>, and NiSO<sub>4</sub>. *E. coli-CynD* was adjusted to an OD<sub>600nm</sub> of 1.1 in 20 mM buffer tris (pH 8.0) and 100 µL were incubated with 200 ppm of the metal for 1 hour. The activity of the metal incubated *E. coli-CynD* cells was determined in 1 mL assays with 5 mM cyanide and 20 mM Tris (pH 8.0) buffer. Activity is reported relative to *E. coli-CynD* cells that were not incubated with any heavy metal salts. All assays were performed in triplicate.

### Bioremediation of industrial cyanide wastewater

Samples of industrial cyanide wastewater were obtained from a gold processing plant belonging to La Sociedad Minera del Sur S.A., Buenos Aires Cauca, Colombia. Total metal content in the samples was analyzed by inductively coupled plasma mass spectrometry (ICP-MS) by the laboratory SGS, Bogotá, Colombia. The concentration of free cyanide in the industrial samples was determined using the picric acid method.

Whole *E. coli-CynD* cells were adjusted to an OD<sub>600nm</sub> of 1.1 in 20 mM Tris (pH 8.0) buffer. From this solution,

100  $\mu\text{l}$  of cells were added directly to 900  $\mu\text{l}$  of the cyanide-containing wastewater and the cyanide content determined after 30 min. Additional reactions were carried out using cyanide wastewater diluted 30-fold with water and either 100  $\mu\text{l}$  or 300  $\mu\text{l}$  of *E. coli-CynD*. Also, 300  $\mu\text{l}$  of a cell resuspension composed of 200  $\mu\text{l}$  *E. coli* BL21(DE3) and 100  $\mu\text{l}$  of *E. coli-CynD* were used as a control for the possible effect of *E. coli* cellular components on inhibiting constituents present in the cyanide waste solution.

#### Cell viability in industrial cyanide wastewaters

Permeabilized and non-permeabilized *E. coli-CynD* cells were added in a 1:10 ratio to 1 mL cyanide wastewater solutions and allowed to grow for 3 days at 25 °C with constant agitation. Ten  $\mu\text{L}$  from the solution were plated on LB-Kanamycin agar plates and the colony forming units (CFUs) determined after 2 days of growth at 37 °C. As a control we did a similar procedure but with *E. coli-CynD* cells grown in LB broth. All experiments were performed in triplicate.

#### Software

Minitab 18 Statistical Software by Minitab © was used for the statistical analysis utilizing a 95% confidence interval. GraphPad Prism 7 by GraphPad Software was used for the kinetics analysis and Graphics construction.

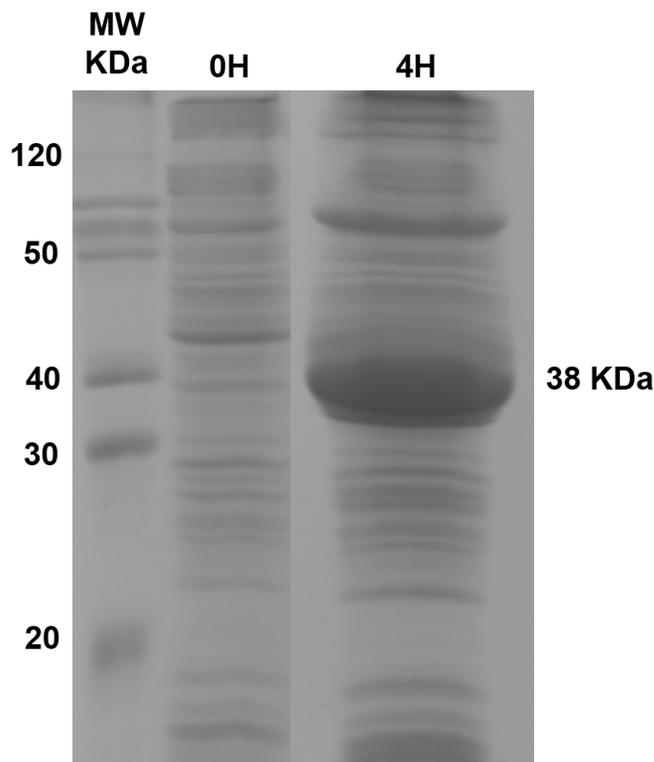
## RESULTS AND DISCUSSION

#### Overexpression of intracellular CynD and kinetics

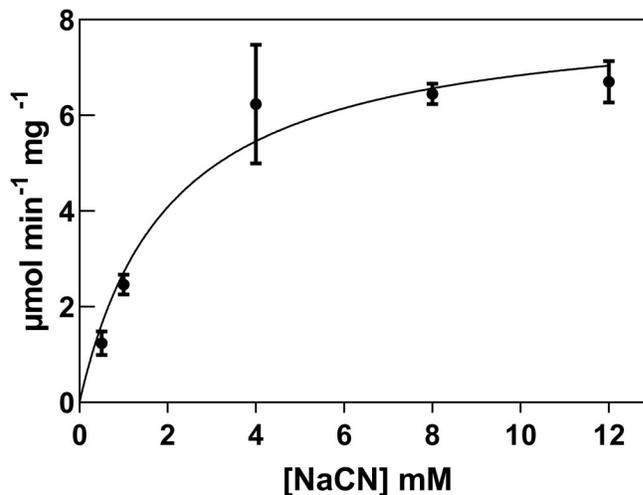
Recombinant CynD expressed abundantly in *E. coli*, with maximal expression at 4 hours after IPTG induction. The prominent band at 38 kDa observed in SDS-PAGE gels of total protein content (Figure 1) corresponds to the protein product predicted by the gene sequence (Jandhyala *et al.*, 2003). *E. coli-CynD* showed saturation kinetics (Figure 2) with values for  $V_{\text{max}}$  of 8.5  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  and  $K_M$  for cyanide of 2.1 mM. These values can be compared with those reported for the purified enzyme ( $V_{\text{max}}$  88  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  and  $K_M$  2.56 mM) (Jandhyala *et al.*, 2005;). The similar  $K_M$  value for intracellular and purified enzyme suggests that cyanide can rapidly diffuse through the cell membrane, rapidly equalizing inner and outer concentrations, a characteristic that can contribute to cyanide cytotoxicity. With regards to  $V_{\text{max}}$ , comparisons with the purified enzyme are impossible due to the unknown concentration of CynD inside *E. coli*. Altogether, these values can be compared with other intracellular CynD enzymes like that of *F. indicum* ( $V_{\text{max}}$  0.33  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  and  $K_M$  5.25 mM) (Kumar *et al.*, 2018).

#### Enzymatic degradation of cyanide by *E. coli-CynD*

Industrial applications of microbes can utilize whole cells in either their growth or resting phase, or as permea-



**Figure 1.** SDS-PAGE gel of the total protein content of *E. coli-CynD*. The band at 38 kDa corresponds to CynD.



**Figure 2.** Dependence of the initial velocity on the cyanide concentration for *E. coli-CynD*. The line represents the fit of the data to the Michaelis-Menten equation.

bilized cells (Ramesh *et al.*, 2016). We examined the activity of different types of cell preparations of *E. coli-CynD* to degrade cyanide (Table 1). Initial velocity results indicated that growing and resting cells are equally

**Table 1.** Effect of the cell resuspension solution over the activity of *E. coli-CynD*.

Cell type	Resuspension solution	Initial velocity ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ )	Cyanide removed (%)
Growing	LB broth	$2.44 \pm 0,04$	$99.07 \pm 0.8$
Resting	20 mM Tris Buffer (pH 8.0)	$2.78 \pm 0,04$	$98.48 \pm 0.7$
Permeabilized	20 mM Tris Buffer (pH 8.0), 5% Toluene, 1 mM EDTA	$1.72 \pm 0,01$	$98.63 \pm 1.3$
Untransformed	20 mM Tris Buffer (pH 8.0)	ND	$0.42 \pm 0.7\%$

active, however, permeabilized cells are less active. Nevertheless, all cells completely depleted cyanide from a 5 mM solution within 10 minutes. In contrast, cyanide removal by untransformed *E. coli* cells was statistically equal to that caused by evaporation ( $0.42 \pm 0,7\%$ ). For the bioremediation of industrial cyanide wastewater, the use of a resting-cell process (Law *et al.*, 2006) where the production of the biocatalysts through fermentation is separated from the biocatalytic process can overcome the cost associated with using complex media for biomass production. For all subsequent experiments reported here, resting (stationary) phase cell cultures were used.

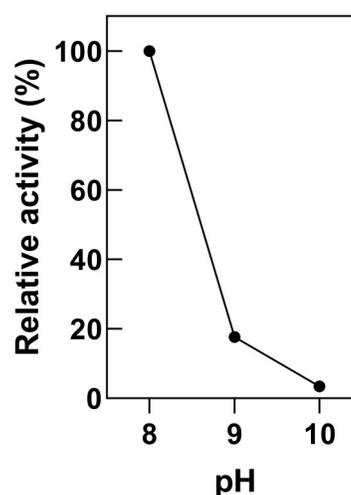
#### ***E. coli-CynD* activity at alkaline pH**

HCN and  $\text{CN}^-$  species exist in a pH-dependent equilibrium where the anion dominates at high pH. Thus, industrial cyanide solutions are maintained at pH 10 or above to avoid the dangerous volatilization of HCN (Johnson, 2015). The activity of resting *E. coli-CynD* cells at pH 8, 9 and 10 was examined (Figure 3). The alkaline pH degraded or inactivated the intracellular enzyme. Still, at pH 9.0 the enzyme retained 20% of the activity. This result contrast with the marked fall-off in activity at pH above 8.5 observed for the purified CynD enzyme (Jandhyala *et al.*, 2005). Nevertheless, the residual activity of intracellular CynD was enough to achieve cyanide removal close to 100% at 20 minutes at pH 9.0 and 78% after 40 minutes at pH 10 (Data not shown).

Recently, protein engineering efforts have produced mutant CynD enzymes that retain activity at pH 9.5 (Crum *et al.*, 2015; Crum *et al.*, 2016). It is plausible that the use of whole cells overexpressing the more pH-resistant forms of CynD could provide a way to extend the pH tolerance of these enzymes to even more alkaline values.

#### **Thermostability of *E. coli-CynD***

Industrial processes that involve cyanide solutions can operate at temperatures above 42 °C, which is a com-



**Figure 3.** Activity of *E. coli-CynD* at alkaline pH. Activity is calculated relative to the activity at pH 8.0. Data represent the mean  $\pm$  sd of three independent experiments.

mon occurrence in tropical countries. Purified recombinant wild-type CynD displays maximum activity between 37 °C and 42 °C (Jandhyala *et al.*, 2005). However, it displays low thermal stability and activity is completely lost after a 5 hour incubation period at 42 °C (Crum *et al.*, 2015; Crum *et al.*, 2016). In contrast, intracellular CynD was more resistant to high temperature and it retained > 60% activity at 50 °C (Figure 4).

Thermal instability is a common feature of cyanide degrading enzymes such as CynD and cyanide hydratase (CHT) (Jandhyala *et al.*, 2005). When incubated at 42 °C, purified wild-type CynD rapidly loses its cyanide-degrading activity (Crum *et al.*, 2015; Crum *et al.*, 2016), and at 55 °C, it becomes completely inactive in a matter of minutes (Jandhyala *et al.*, 2005). In contrast, intracellular CynD remains fully active at 42 °C for up to 72 h and retains 30% of its activity at 60 °C over the time

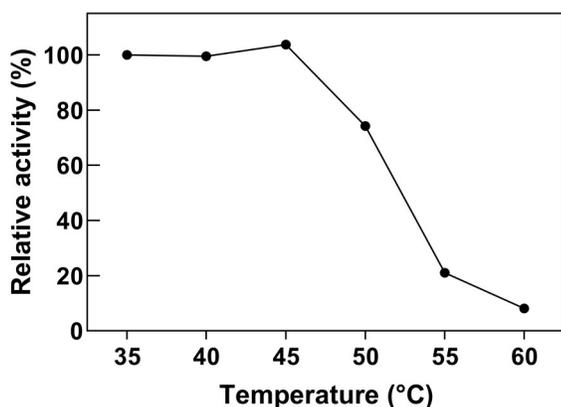


Figure 4. Optimal temperature range for *E. coli-CynD*. Relative activities were calculated with respect to the activity at 35°C.

course in which the purified enzyme becomes completely inactive (Figure 5). The thermostability of intracellular CynD even surmounts that of the most thermostable CynD chimera produced through protein engineering efforts (Crum *et al.*, 2015). The mutant enzyme formed by swapping the C-terminal sequence of CynD from *B. pumilus* with that of *P. stutzeri* AK61 displayed less than 20% residual activity after 42 h at 42°C; whereas *E. coli-CynD* remained fully active within the same time (Figure 5). Similar patterns for the protection of enzyme activity are common when using whole cells to overexpress thermolabile enzymes. Furthermore, these approaches highlight the benefits of using whole cells for industrial applications in environments that are unfavorable for the activity of the enzyme (Wachtmeister & Rother, 2016).

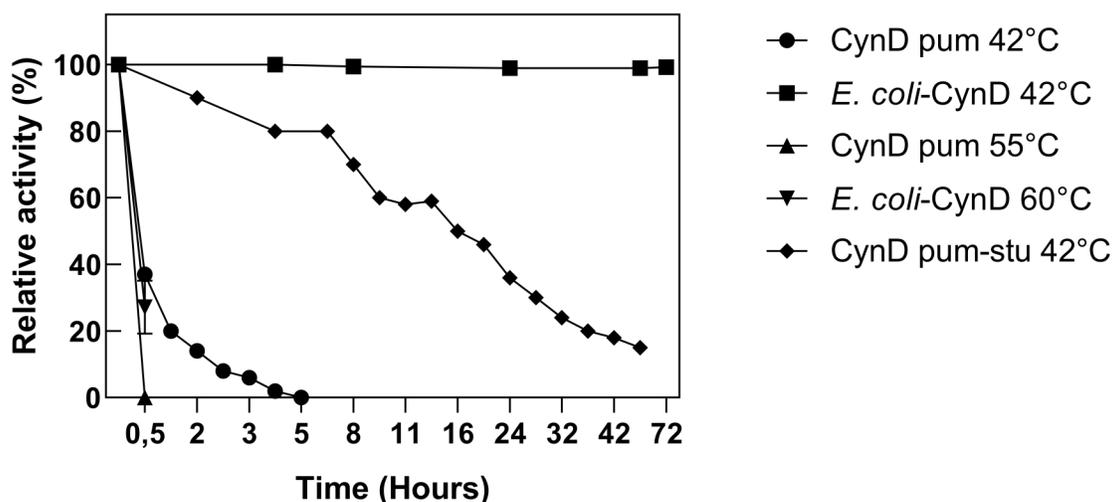


Figure 5. Thermostability of *E. coli-CynD*. For comparison, data for purified CynD (CynD pum) and the most thermostable chimera (CynD pum-stu) are shown (M. A. Crum *et al.*, 2015; Mary A. Crum *et al.*, 2016).

### Heavy metal inhibition of *E. coli-CynD*

Purified CynD enzyme is strongly inhibited by  $Hg^{2+}$  and  $Pb^{2+}$  (D. M. Jandhyala *et al.*, 2005). Analysis of the metal content of a solution obtained from a gold mine in Colombia revealed that  $Fe^{2+}$ ,  $Hg^{2+}$ , and  $Ni^{2+}$  were abundant. Therefore, cyanide removal by *E. coli-CynD* was determined in separate solutions of these metals (Figure 6). In all cases, the metal concentrations tested were higher than those found in the solution obtained from the gold mine. The level of inhibition of *E. coli-CynD* was less pronounced than observed for the purified enzyme where cyanide-removal activity falls below 10% (Jandhyala *et al.*, 2005).

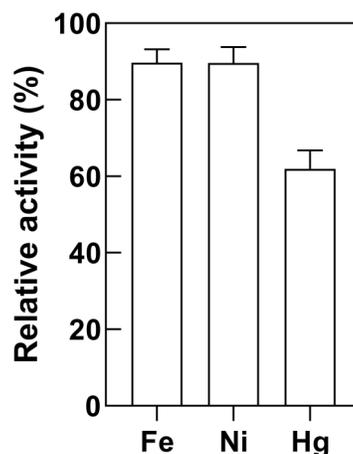
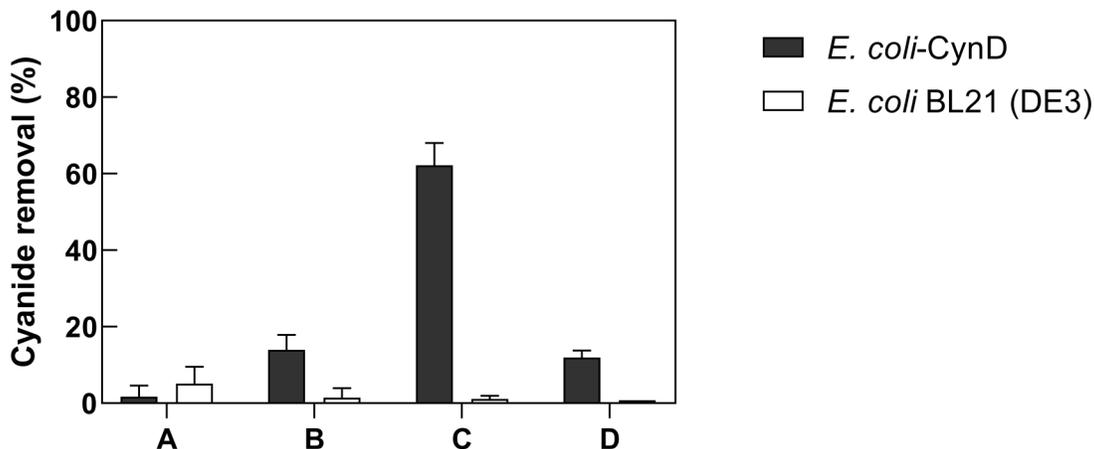


Figure 6. Comparison of the relative cyanide-removal activity of *E. coli-CynD* in the presence of 200 ppm  $Fe^{2+}$ ,  $Hg^{2+}$ , or  $Ni^{2+}$



**Figure 7.** Ability of *E. coli-CynD* to remove free cyanide from gold mine wastewater. A. 1:10 dilution of *E. coli-CynD* cells to cyanide solution. B. 1:10 dilution of *E. coli-CynD* cells to a 30-fold dilution of the cyanide solution. C. 3:10 dilution of *E. coli-CynD* cells to a 30-fold dilution of the cyanide solution. D. 1:2:10 dilution of *E. coli-CynD* cells, untransformed *E. coli* cells to a 30-fold dilution of the cyanide solution.

### Bioremediation of gold mining cyanide wastewaters

Gold mine derived cyanide wastewater is maintained at a very alkaline pH (>11). Additionally, it also contains high cyanide concentrations and variable concentrations of heavy metals and minerals. Therefore, the ability of *E. coli-CynD* to degrade free cyanide in wastewaters collected from a Colombian gold processing plant was tested (Figure 7). Results indicated that the intracellular CynD was completely inhibited when cells were added directly to the mine wastewater (Figure 7, Treatment A).

Therefore, the wastewater solution was diluted 30-fold with water. The *E. coli-CynD* cells, however, could only degrade 14% of the free cyanide in that diluted solution (Figure 7, Treatment B). However, when the concentration of cells added increased by three-fold, cyanide degradation in the diluted solution was almost 70% (Figure 7, Treatment C). A possible explanation for the increase in cyanide-removal activity under the latter conditions is the potential interaction of cellular components with the inhibiting molecules present in the cyanide solution.

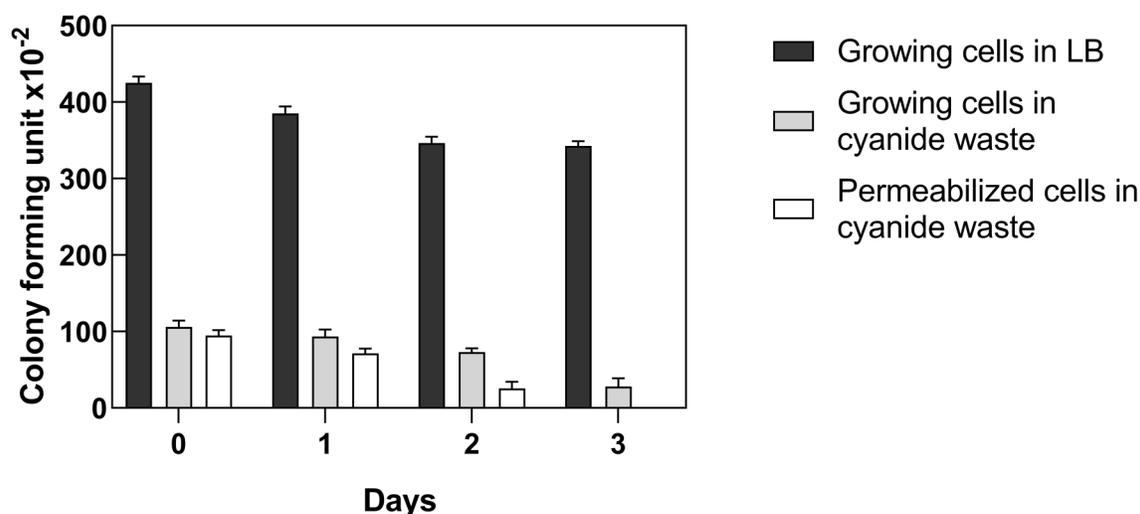
The results of treatment D (Figure 7) suggest that it is not any endogenous *E. coli* cellular component that prevents the inhibition, but rather that CynD itself is the molecular entity which chelates the inhibitors present in the wastewater solution. This hypothesis is supported by the fact that when untransformed *E. coli* cells are added together with *E. coli-CynD* cells, cyanide-removal activity only reached a level observed with cells expressing CynD. Similar results were obtained with *E. coli-CynD* cells and *E. coli* cells transformed with an “empty” plasmid.

A further explanation for the prevention of inhibition when higher concentrations of transformed cells are used is that, if heavy metals in the cyanide waste solution are the source of inhibition, a portion of the overexpressed enzyme could bind to the heavy metal compounds and still leave enough “free” enzyme to exert catalysis of free cyanide. A conserved cysteine-glutamate-lysine triad characterizes enzymes of the nitrilase super family (Park *et al.*, 2017). Therefore, the catalytic triad in CynD could act as metal chelator. The strong inhibition of the purified enzyme by Hg<sup>2+</sup> and Pb<sup>2+</sup> (Jandhyala *et al.*, 2005) supports our hypothesis.

Our results, using *E. coli-CynD* demonstrated that by adjusting the ratio of live cells to dilutions of the cyanide-containing effluent, biodegradation of the free cyanide in the solution can be achieved. Moreover, these results encourage the use of a bioreactor based on *E. coli-CynD* cells. Cyanide effluents in this bioreactor could be circulated, until legal cyanide limits for disposal are met. In the present study only free cyanide degradation was determined. Experiments designed to determine the degradation of total cyanide (WAD or SAD cyanide) by *E. coli-CynD* are in progress.

### *E. coli-CynD* viability in gold mining cyanide wastewaters

Strict regulations exist for the use of recombinant microorganisms in open environments, such as gold processing plants. *E. coli-CynD* is a recombinant organism and concerns may exist about their fate in the environment. Therefore, the cellular viability of recombinant *E. coli-CynD* cells in the industrial cyanide waste solution



**Figure 8.** Cellular viability of exponential and resting *E. coli-CynD* cells in gold processing cyanide waste solution. Colony forming units (CFU) were counted on LB agar plates after culturing for 16 hours at 37°C.

was determined (Figure 8). Results indicated that permeabilized *E. coli-CynD* cells lose their viability after three days in the gold mine processing wastewater. The use of permeabilized cells, however, is undesirable due to the need to use organic solvents to produce them. Therefore, bioreactors utilizing immobilized *E. coli-CynD* on solid supports (Carmona & Panay, 2019) should be explored for the bioremediation of industrial cyanide wastes.

## CONCLUSION

The use of whole *E. coli* cells overexpressing CynD presents an improvement over the thermostability of the purified CynD enzyme. The capacity of the intracellular enzyme to retain > 60% activity at 50 °C is a quality that can be exploited for the immobilization of the catalyst in thermogels like agarose. Also, improvement was observed in the pH resistance of the intracellular enzyme. Finally, our results indicated that *E. coli-CynD* is a viable alternative for the biodegradation of free cyanide in harsh industrial solutions like those derived from the mining industry.

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