Cyano-Derivative Degradation by *Pseudomonas fluorescens*: A Biochemical Study

By

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Approved by:

Supervisor: __________________________ Date: ______________________

Second Reader: _______________________ Date: _______________________
Abstract

Thiocyanate (SCN) and cyanate (OCN), toxic derivatives of cyanide (CN), are often produced as waste products within the mining industry. Both SCN and OCN elicit neurodegenerative symptoms in various species of mammals and fish, and can rapidly lead to death. Mining effluents often contain SCN/OCN, and current decontamination technologies are expensive, time-consuming, and/or ineffective. Bioremediation, the process of eliminating pollutants by living systems, offers an attractive route to neutralise both compounds, as the sulphur and nitrogen are essential for cellular growth. *Pseudomonas fluorescens*, a versatile and resilient soil microbe, has potential for use in bioremediation. In this study, *P. fluorescens* was grown using OCN as sole nitrogen source, and SCN as sole nitrogen and sulphur source. *P. fluorescens* was able completely degrade 10 mM of OCN and SCN in 24 and 122 hours, respectively. Cyanase, an enzyme involved in the degradation of both cyano-derivatives, was detected in membrane fractions in both treatment groups. *P. fluorescens* was unable to degrade SCN in the presence of free sulphate, suggesting that low sulphur conditions are required in order to induce expression of SCN degrading enzymes. *P. fluorescens*’ ability to mineralize cyano-derivatives at relatively high concentrations indicates that it would be well suited to use within industrialized bioremediation.

**Key Words:** Cyano-Derivatives, Thiocyanate, Cyanate, Cyanase, *Pseudomonas fluorescens*, Bioremediation
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## Abbreviations

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<th>Description</th>
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<tr>
<td>BN-PAGE</td>
<td>Blue-Native Polyacrylamide Gel Electrophoresis</td>
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<td>CN</td>
<td>Cyanide</td>
</tr>
<tr>
<td>COS</td>
<td>Carbonyl Sulfide</td>
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<tr>
<td>DCPIP</td>
<td>2,6-dichlorophenolindophenol</td>
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<tr>
<td>GDH</td>
<td>Glutamate Dehydrogenase</td>
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<tr>
<td>HCN</td>
<td>Hydrogen Cyanide</td>
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<tr>
<td>HCO$_3$</td>
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<td>TcDH</td>
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Chapter 1 Introduction

1.1 Cyanide in Industry

Cyanide (CN) has garnered a reputation as an extremely potent toxicant, with good reason; the LD$_{50}$ in humans has been estimated to be around 1.00 mg/kg IV and 1.52 mg/kg orally [1,2]. Despite its toxic effects, CN has many uses in industry and production, and is still in use today. CN is often formed as a by-product in the production of many compounds, including the synthesis of organic nitriles, acrylic plastics, paints, dyes, drugs, and chelating agents [3]. While these industries produce large quantities of CN by-products, the largest contributors are the mining industry and steel production, with gold mining and precious metal extraction using nearly 6% of all produced cyanide [4,5]. Within the jewellery industry, cyanide is used to extract precious metals, such as gold and silver, from the wastewaters generated after electroplating [5]. In the case of gold mining, ore containing gold is ground and mixed with CN, which extracts the gold from the ore [6]. CN reacts with gold via the Elsner equation (1), binding with high affinity and dissolving gold from the ore slurry [6].

Equation 1

\[ 4\text{Au} + 8\text{CN}^- + \text{O}_2 + 2\text{H}_2\text{O} \leftrightarrow 4\text{Au(CN)}_2^- + 4\text{OH}^- \]

An excess of zinc is then added to the solution, dissociating the gold from CN, and allowing the gold to precipitate [7]. The remaining wastewater can then be recycled, allowing the CN to be reused for further gold extraction cycles, or is stored in mine tailings while undergoing decontamination treatment prior to release into the environment [8].
1.2 Cyano-Derivative By-Products

The use of CN in industry, particularly when formed as a by-product, can lead to the generation of several cyanide related compounds. This includes metal-cyanide complexes and cyano-derivatives [9]. The cyano-derivatives commonly found alongside CN in waste waters are thiocyanate (SCN) and cyanate (OCN). SCN is considered the more toxic of the two, and is often generated as a result of CN reaction with reduced sulphur species within solution [3]. This is particularly frequent in the context of gold extraction, as sulphur species are often present in gold containing ore, and can form anywhere between 40-600 mg/L (0.70-10.33 mM) of SCN [10]. In contrast, cyanate is often deliberately produced in an effort to detoxify CN, as it is considered to be nontoxic [11]. The exact method through which the cyano-derivatives are generated is irrelevant, as they are both commonly found in mine effluents, along with many other toxic compounds including sulphate, ammonia, nitrate, and trace quantities of free CN [12].

1.3 Cyano-Derivative Toxicity

The potent toxicity of CN comes from its ability to bind and inhibit cytochrome c oxidase, a vital component of cellular respiration [1,2]. This prevents the use of oxygen for energy production, leading to hypoxia throughout the body, and at high enough doses, death [1]. In contrast to CN, SCN and OCN have no reported effects on cellular respiration in vivo, and have a variety of effects depending on the organism.

The mechanism of OCN toxicity is the better understood of the two cyano-derivatives. OCN poisoning is known to produce a variety of neuropathy related symptoms, including psychosis, delirium, hallucinations, convulsions, coma, and in severe cases, death [13]. OCN
poisoning has been linked to the demyelination of neurons, specifically in the pyramidal tracts of the spinal cord [14]. The myelinotoxic effects of cyanate have been attributed to the effects of carbamylation, wherein OCN reacts with free nitrogen at the N-terminal of a protein, leading to damage and possibly degradation [14,15]. OCN has also been shown to act as an inhibitor of cytochrome c oxidase, however this has only been shown in vitro, and has not been proven to act in vivo and contribute to the toxicity of OCN [16].

SCN toxicity exhibits many of the same physiological symptoms in mammals as OCN, such as psychosis, convulsions, and coma, and it has therefore been proposed that the two cyano-derivatives share a common mechanism of action; however, there is currently no evidence supporting demyelination due to SCN [13,17]. Both cyano-derivatives have been implicated in lethality of freshwater trout [18]. The effects seen in trout after cyano-derivative poisoning are summarized as “sudden death syndrome”, which is characterized by convulsions, loss of buoyancy, darkening of epithelium, respiratory arrest, and extreme rigor [12,18–21]. The LC$_{50}$ of SCN in rainbow trout has been estimated to be around 177-264 mg/L in water, while OCN has a lower LC$_{50}$ of 15-81 mg/L [18]. The values differ due to various factors including pH, temperature, and stress levels during and after poisoning.

SCN has also been shown to have severe effects on trout eggs as well, while data on the effects of OCN on trout eggs was unavailable at the time of this writing. Specifically, SCN concentrations of 2,700 mg/L or greater lead to significant egg mortality, while eggs that survived after exposure to 85 mg/L or greater often developed severe deformities of spine and tail [20].
1.4 Environmental Contaminations

In developed nations, regulations regarding CN and other toxic compounds released into the environment are strict. In Canada, for instance, effluent with concentrations of any deleterious compounds above the authorized limit must be detained in a disposal area which is confined, either naturally or anthropogenically, so long as it is not a natural body of water frequented by fish [8]. If the concentrations of all deleterious compounds are below said value, the effluent can be freely disposed of in any water. The limit for CN, which accounts for all forms of CN in solution, including SCN and OCN, is 1.00 mg/L as a monthly mean [8]. These strict regulations, in addition to monthly acute lethality testing of effluent on rainbow trout, ensure that environmental contamination from mine effluent in Canada is drastically reduced [8]. Unfortunately, industries in less developed nations are far less strict, and the high cost associated with effluent decontamination leads to the improper decontamination of effluents prior to disposal [22]. In addition, effluents containing toxic levels of deleterious compounds stored in tailings prior to or during decontamination are a potential hazard, as mine tailings have leaked, collapsed, or overflowed in the past [23,24]. As a result, decontamination techniques must be developed that are affordable and easily implemented, act to completely degrade a variety of compounds, and do so in a relatively quick manner.

1.5 Current Decontamination Techniques

Current mining effluent decontamination techniques prioritize the removal and degradation of CN, while other toxic cyano-derivatives, including SCN and OCN, are overlooked [12]. SCN is typically considered to be a priority for decontamination, however OCN is not considered a threat to the environment, and is often deliberately formed as the
end product in CN decontamination [6,11,12]. The most common technique for removing SCN from effluents involve the conversion back into CN, allowing the CN to be re-used for further gold-leaching cycles, or through oxidation to OCN [10,12]. This procedure typically involves the oxidation of SCN by treatment with ozone, which when done at low pH leads to the regeneration of CN [10,12]. Done under alkaline conditions, this technique can lead to the over oxidation of CN into OCN, and could therefore be used to ‘detoxify’ CN. Alternate methods for CN detoxification, rather than regeneration, are the INCO SO₂/Air process, the use of Caro’s acid, and the use of hydrogen peroxide. All three techniques show limited efficacy at oxidizing SCN, and the primary product formed is OCN [11]. The optimal operating pH for all of these techniques is 8 or higher, primarily because the basic pH prevents the production of hydrogen cyanide gas (HCN), which is less responsive to treatment, and is deadly if released [11,12,25]. While these chemical detoxification techniques are successful at removing CN from the system, they fail to completely degrade the toxic compounds, and consider OCN to be a safe and satisfactory product. The belief that OCN is safe for disposal into the environment is clearly false, and techniques for the effective removal of all cyano-derivatives should be considered.

1.6 Bioremediation and Cyanide

An alternative to chemical decontamination is biological decontamination. This process is typically referred to as bioremediation, wherein microorganisms are employed to degrade, detoxify, mineralize, or otherwise remove pollutants from a system [26]. Bioremediation occurs naturally during environmental pollution, but can be deliberately used in effort to produce a relatively cheap and environmentally friendly approach to degrade
contained pollutants. In mining, a desire for a more efficient method for complete toxicant degradation has resulted increasing interest in bioremediation.

Multiple strains of bacteria have been isolated from environments exposed to environmental contaminants, including CN, and their efficacy as cyanotrophs has been studied [3,27,28]. These cyanotrophs, thusly named for their ability to degrade and consume CN and cyano-derivatives, have been extensively studied to determine their mechanisms of action [3,5,12,23,27,29]. Two major pathways for SCN degradation have since been uncovered (Figure 1.1). The first pathway, named the Carbonyl Sulfide Pathway, involves the degradation of SCN into carbonyl sulfide (COS) by the enzyme thiocyanate hydrolase (SCNase) (Equation 2), which is then further mineralized into carbon dioxide and sulphate by nitrogenase (Equation 3) [12].

**Equation 2**

\[
\text{SCN}^-+2\text{H}_2\text{O} \rightarrow \text{COS} +\text{NH}_3+\text{OH}^-
\]

**Equation 3**

\[
\text{COS}+\text{OH}^-+2\text{O}_2 \rightarrow \text{CO}_2 +\text{SO}_4^{-2}+\text{H}^+
\]

The second pathway, also named for the intermediate formed, is the Cyanate Pathway. This pathway differs from the COS pathway only in the intermediate species formed, as the final products of CO\(_2\), NH\(_3\), and SO\(_4\) remain constant (Figure 1.1) [12]. The first reaction in this pathway is catalyzed by SCN dehydrogenase (TcDH), and releases SO\(_4\)\(^{-2}\) (Equation 4) (Figure 1.1). The second enzyme, cyanase, catalyzes the degradation of OCN into ammonium and bicarbonate, which can be incorporated into the bacteria’s metabolism (Equation 5).
The Cyanate Pathway (left) and the Carbonyl Sulfide Pathway (right) are the two known enzymatic pathways for SCN consumption in bacteria. Both pathways yield the same products of sulphate, ammonia, and carbon dioxide. The enzymes involved in these reactions are denoted in red, alongside the reaction they catalyze.

**Equation 4**

$$\text{SCN}^- + \text{H}_2\text{O} + 2\text{O}_2 \rightarrow \text{HCNO} + \text{SO}_4^{2-} + \text{H}^+$$

**Equation 5**

$$\text{HCNO} + 2\text{H}_2\text{O} \rightarrow \text{NH}_4^+ + \text{HCO}^-$$

While the general scheme of these enzymatic reactions holds true, there is some variation in the reported sulphur species formed. For example, Mason et al. report the COS Pathway microbes release hydrogen sulfide, not sulphate, as their primary sulphur product [30]. The most likely cause for these variations is the presence of additional enzymes which immediately facilitate conversion into other species, which are then accumulated or stored within the cells, and is entirely dependent on physiological needs.
1.7 *Pseudomonas* and Cyanide

Many different species of microbes have been studied in hopes of discovering effective cyanotrophs. One microbe of interest is the common soil bacterium, *Pseudomonas fluorescens*. *P. fluorescens* is a rod-like Gram-negative bacterium, and is found practically everywhere, including in soil, the rhizospheres and surfaces of plants, on indoor walls, and on humans, most commonly in the mouth and lungs [31]. Given the bacterium’s ability to survive in a diverse spectrum of environments and the fact that it is nonpathogenic, its ability to act as a cyanotroph should be examined, as it could be readily implemented within bioremediation of industrial effluents [31].

A previous study showed that *P. fluorescens* is able to degrade SCN, and even suggested that this occurs through the Cyanate Pathway [30]. Further, several other strains of *Pseudomonas* have shown ability to act as cyanotrophs, including *pseudoalcaligenes, aeruginosa, putida, stutzeri,* and *paucimobilis*; however, the efficacy and mechanisms used by *P. fluorescens* to mineralize cyano-derivatives are poorly understood. This study aims to further examine the role *P. fluorescens* could play in bioremediation, by exploring its ability to mineralize the major cyano-derivatives under various physiological conditions.
Chapter 2 Objectives and Hypothesis

The objective of this study was to conduct exploratory studies on the efficacy of *Pseudomonas fluorescens* as a cyanotroph under a variety of conditions, including varying pH, carbon sources, and cyano-derivative concentrations. The study aimed to show that *P. fluorescens* is capable of degrading OCN as sole nitrogen source in the presence of free sulphur, and that SCN can be used as both sole nitrogen and sulphur sources. Furthermore, the study aimed to observe physiological and enzymatic changes within the bacteria in response to cyano-derivative stress, including growth, pH, intra and extra-cellular metabolites, and enzymatic activity, particularly within the pathways associated with cyano-derivative decomposition. *P. fluorescens* may be used efficiently and effectively used as a cyanotroph within industrialized bioremediation, as the microbe is versatile and resilient, and diverse in its nutritional resources.
Chapter 3 Materials and Methods

3.1 Bacteria Strain and Cell Culture

*Pseudomonas fluorescens* (ATCC, Manassas, VA) was grown in preculture citrate mineral media for 24 hours and was kept up to 7 days. Citrate media contained 0.4 mM NaHPO₄, 0.22 mM KH₂PO₄, 0.811 mM MgSO₄·7H₂O, 15 mM NH₄Cl, and 19 mM citrate. Trace elements were present in concentrations as described previously [32]. After reaching stationary phase, 5 mL of preculture *P. fluorescens* was added to media containing the required cyano-derivative and carbon source concentrations. All cyano-derivative stress and control media was prepared with preculture concentrations of citrate, NaHPO₄, and KH₂PO₄, while 10 mM NH₃ was added via OCN, SCN, or NH₄Cl. SCN media were not supplied with additional sulphur; as such, 0.811 mM MgCl₂ was added in place of MgSO₄.

3.2 Culture Growth and Sampling

Cultures were prepared with 200 mL of media in a 500 mL Erlenmeyer flask inoculated with 5 mL of stationary phase preculture cells, and grown in an aerated gyratory water bath shaker, model 76 (New Brunswick Scientific) at 25 °C at 140 rpm until reaching stationary phase. Every 12/24 hours 10 mL aliquots were taken, and were then centrifuged at 10,000 × g for 20 minutes to separate cells (pellet) from spent fluid (supernatant). From these samples 1 mL aliquots of spent fluid were stored at -20°C for metabolite quantification. Cells were resuspended in 0.5 M NaOH and heated at 80-100°C for 10 minutes to denature and hydrolyze proteins. Denatured proteins were then quantified using the Bradford method to approximate cell number and culture growth.
3.3 Protein Quantification

The Bradford method was used to quantify protein levels and biomass, using bovine serum albumin as a standard [32–34]. Briefly, 200 μL of Bradford Reagent (B6916, Sigma-Aldrich) were added to 20-100 μL of denatured protein sample (see 3.2), completed to 1 mL with ddH₂O, incubated for 3 minutes, and the absorbance recorded at 595 nm using a Thermo Scientific Genesys 30 Visible Spectrophotometer. Using a BSA standard curve prepared under the same conditions, the protein concentration in resuspended pellet was determined, and used to calculate the total protein concentration in culture. The total protein concentration, or the biomass, found within culture is largely dependent on the number of cells present, and therefore reflects overall cell numbers, and can be used to quantify growth.

3.4 Metabolite Quantification

3.4.1 Citrate/Acetate/Glycerol/SCN

Metabolites which absorb light between 200-500 nm could be quantified using reverse-phase HPLC with C18 column equipped with a dual wave-length detector (Waters 2695 Separations Module, Waters 2487 Dual λ Absorbance Detector). Metabolites which were quantified by HPLC include citrate, acetate, and glycerol. HPLC was also used as a secondary confirmation of SCN levels. Samples were prepared by filtering 100 μL of spent fluid through a glass pipette with glass wool, followed by elution with 900 μL ddH₂O. Samples were then run in HPLC for 20 minutes at a flow rate of 0.7 mL/minute, with a buffer consisting of 20 mM KH₂PO₄ at pH 2.9. Metabolites were quantified at 210 nm, and data was analyzed using Empower software by quantifying the area under the curve of the metabolite’s peak. The elution time and identity of metabolites was confirmed by running 30
μL of 100 mM standards diluted to 1 mL concurrently with samples, and by spiking samples with 1 μL standard and rerunning through HPLC to monitor changes in peak area.

3.4.2 OCN

OCN levels were determined through hydrolysis followed by quantification of released ammonia, as described previously [35]. This procedure was modified to quantify only free ammonia, by maintaining the reagents at pH 7. OCN showed no absorbance when treated at pH 7 (data not shown). Samples were quantified using both sets of reagents (pH 3, pH 7), generating free ammonia and total ammonia concentrations. Given that 1 mol OCN approximately yields 1 mol NH₃, the free ammonia concentration was subtracted from the total ammonia concentration, giving the concentration of OCN.

3.4.3 SCN

SCN was quantified through reaction with iron, forming a red complex which could be quantified through absorbance at 460 nm. The procedure was adapted from previous studies, and is summarized below [36]. A 500 μL of a ferric nitrate solution containing 0.143 M Iron (III) nitrate nonahydrate and 9.31% (w/w) nitric acid was reacted with 25-100 μL of sample, and completed to 1 mL with ddH₂O. Samples were incubated for 3 minutes then absorbance was quantified at 460 nm.

3.5 Fraction Isolation/Preparation

After cultures reached stationary phase (Figure 3.1), the entire culture was centrifuged at 10,000 × G for 20 minutes. A 10 mL aliquot of spent fluid was stored at -20
and the pellet was washed with 0.85% (w/v) NaCl solution with an additional 20 minutes
spin. After

![Figure 3:1 Cultures at Stationary Phase](image)

Control and OCN Stress cultures at stationary phase, respectively.

decanting the supernatant, the pellet was resuspended in 1 mL of cell storage buffer (CSB; 50 mM Tris-HCl pH 7.6, 5 mM MgCl₂, 1 mM dithiothreitol and 1 mM phenylmethylsulphonyl fluoride (PMSF)). The cells were then lysed using sonication and
centrifuged at 180,000 × g for 3 hours, resulting in soluble cell free extract (CFE) and
membrane CFE. The fractions were stored at 4 °C for up to 5 days, and various enzymatic
activities were monitored.

3.6 Enzyme Activity

Protein activity was quantified using Blue Native PAGE (BN-PAGE) as described
previously [32,34,37]. A 4-16% gradient gel was cast with the Bio-Rad Model 385 Gradient
Former, and the Bio-Rad MiniProtean™ 2 system using 1 mm spacers. Cellular fractions
containing 60 μg total protein were loaded onto the gel and blue cathode buffer (50 mM
Tricine, 15 mM Bis-Tris, 0.02% (w/v) Coomassie G-250, pH 7, 4 °C) was used during the first half of the run. After the front reached half the length of the gel, the buffer was changed to a colourless cathode buffer (50 mM Tricine, 15 mM Bis-Tris, pH 7, 4 °C) to allow for better visualization. The visualization of protein activity was conducted by coupling the production of oxidizing or reducing factors of the native enzymes to the production of formazan. This was achieved by adding phenazine methosulfate (PMS) and iodonitrotetrazolium (INT) for reducing cofactors, or 2,6-dichlorophenolindophenol (DCPIP) with INT for oxidizing cofactors (Figure 3.2) [37]. Several enzymes were tested using BN-PAGE coupled formazan precipitation, including isocitrate dehydrogenase, glutamate dehydrogenase, TcDH, cyanase. The reaction mechanism for cyanase detection is shown, as it was the only enzymatic data presented (Figure 3.3).

3.7 Statistical Analysis and Data Presentation

All data presented was given as mean ± standard deviation. All experiments were conducted with n=2. Due to the low number of replicates, no proper statistical analysis could be conducted.
Figure 3:2 Schematic of Enzyme Visualization via Formazan Precipitation
(from Auger et al, 2015) [37]

Figure 3:3 Coupled Enzyme Reaction Quantification of Cyanase Activity in BN-PAGE
Coupled enzyme reactions used for cyanase activity visualization in BN-PAGE
Chapter 4 Results

4.1 Cyanate as Sole Nitrogen Source

Media was prepped as indicated in section 3.1, with the nitrogen content in both control and stress media being lowered to 10 mM of NH₄Cl or NaOCN, respectively. OCN cultures were grown with an initial pH 7. Cultures were grown until reaching stationary phase, at around 24 hours (Figure 4.1, 4.2). OCN was fully degraded in 24 hours (Figure 4.2). Control conditions reached stationary phase in just 12 hours, while OCN stress cells required a full 24 hours to reach stationary phase (Figure 4.1).

BN-PAGE was conducted to visualize the enzyme responsible for OCN consumption, believed to be cyanase (Figure 1.1). As such, membrane and soluble CFEs from both control and stress cells were run on BN-PAGE. In order to visualize activity, the production of NH₃ by cyanase was coupled to the conversion of α-ketoglutarate to glutamate by glutamate dehydrogenase (GDH) (Figure 3.3). The resulting formazan bands show stronger enzymatic activity in the membrane fractions, and further cyanase studies were conducted solely in membrane CFE fractions (Figure 4.3). Interestingly, in every BN-PAGE activity assay conducted, control cultures were found to have high levels of cyanase activity, comparable to the stress cultures (Data not shown).

Membrane CFE fractions were treated with 2 mM OCN, and OCN and NH₃ levels were quantified (Figure 4.4). Total NH₃ present is shown, with OCN hydrolyzed ammonia represented by the dark grey bar and free ammonia represented by the light grey. At 0 hour, less than 1 % of the ammonia present in the reaction was free prior to hydrolysis, while after
1 hour 73% of all ammonia was free, and only 27% of the ammonia present was freed from OCN by hydrolysis.

An exploratory culture using elevated concentrations of OCN as sole nitrogen and carbon source was conducted, however no growth was observed within the flask after 14 days, and the culture was subsequently discarded. Additional cultures using in acetate or glycerol were grown, however due to lack of repeats the data is not included.
Figure 4: P. fluorescens Growth Under Control and OCN Stress Conditions

Cultures were grown in 10 mM NH₃, from either NH₄Cl (Control) or OCN (Stress). All cultures were grown at initial pH 7. Biomass (mg/mL) was quantified every 12 hours. Cultures exposed to OCN stress required 12 hours longer to reach stationary phase relative to control cultures. Data was presented as mean ± SD.
Figure 4:2 Complete OCN Mineralization by *P. fluorescens*

Cultures were grown in 10 mM OCN, pH=7, and monitored until reaching stationary phase, near 24 hours. Remaining citrate and OCN (%) and biomass (mg/mL) were quantified at 12 hour intervals. *P. fluorescens* is able to fully degrade 10 mM OCN in the absence of alternate nitrogen sources. Data was presented as mean ± SD.

Figure 4:3 Soluble and Membrane Fractions of Cyanase in OCN Stress Cells

Soluble and Membrane fractions (left and right, respectively) were run on BN-PAGE and reacted to quantify cyanase activity. Both samples were taken from cells grown using OCN as sole nitrogen source. The bar indicates the splicing of two separate images.
Figure 4:4 Membrane CFE OCN Reaction

Approximately 2 mM OCN was added to a membrane CFE mixture containing 200 μg protein. Samples were taken immediately after addition of OCN and 1 hour after reaction start. Samples were heated at 100 °C to deactivate cyanase, and were stored at -20 °C for later quantification. OCN hydrolyzed NH$_3$ and free NH$_3$ levels were quantified as described (3.4.2), and their relative composition at time 0 and 1 hour are shown above.
4.2 Thiocyanate as Sole Sulphur and Nitrogen Source

Media was prepped as indicated in section 3.1, with the nitrogen content in both control and stress media being lowered to 10 mM of NH₄Cl or NaSCN, respectively. In addition, SCN was the sole source of sulphur under stress conditions. Media was prepared at pH 10, as at neutral or acidic pH SCN can form hydrogen cyanide. These cultures were grown until reaching stationary phase, which required between 72-144 hours, after which they were centrifuged and stored for enzyme activity studies (Figure 4.5). Cultures using SCN as sole sulphur and nitrogen source required 122 hours to completely degrade SCN (Figure 4.6), and nearly 140 hours to reach stationary phase, while NH₄⁺ control cultures required on 72 hours (Figure 4.5).

Proper visualization of TcDH through BN-PAGE was unsuccessful; however cyanase was visualized in cells treated with SCN. A slight increase in enzyme activity is visible in SCN treated cells relative to control (Figure 4.7).
Figure 4:5 *P. fluorescens* Growth Under Control and SCN Stress Conditions

Cultures were grown in 10 mM SCN (Stress) or NH$_4$Cl (Control), and monitored until reaching stationary phase. Biomass (mg/mL) was quantified at ~24 hour intervals. Cultures exposed to SCN stress required ~72 hours longer to reach stationary phase relative to control cultures. Data was presented as mean ± SD.
Figure 4:6 Complete SCN Mineralization by *P. fluorescens*

Cultures were grown in 10 mM SCN, and monitored until reaching stationary phase, near 140 hours. Remaining citrate and SCN (%) and biomass (mg/mL) were quantified at ~24 hour intervals. *P. fluorescens* is able to completely mineralize 10 mM SCN within 140 hours. Data was presented as mean ± SD.

Figure 4:7 Membrane Cyanase Activity in Control and SCN Stress Cells

Membrane CFE samples from control (left) and SCN stress (right) cells were run on BN-PAGE and reacted to quantify cyanase activity. Cyanase activity is present in both control and SCN stress cultures with a slight increase in activity in SCN stress.
4.3 Cyanate and Thiocyanate

Cultures were grown under OCN stress with either 5 or 0.5 mM SO$_4$ as sulphur source, and upon reaching stationary phase, were then spiked with an additional 10 mM SCN. SCN concentrations were then monitored at ~12 hour intervals until 48 hours after addition of SCN. Within 24 hours SCN had decreased by approximately 0.5 mM in the low sulphur cultures, while the high sulphur cultures showed no change in SCN levels.
Figure 4:8 High SO\textsubscript{4} Concentrations Reduce SCN Consumption by Stationary Phase Cultures

Cultures were grown in 10 mM OCN, and upon reaching stationary phase, 10 mM SCN was added to the culture. Samples were taken and SCN levels were quantified to visualize SCN consumption. In the presence of 0.5 mM SO\textsubscript{4}, approximately 0.5 mM SCN was consumed by stationary phase cultures. Data was presented as mean ± SD.
Chapter 5 Discussion

Determining the efficacy of *P. fluorescens* as a cyanotroph is the primary focus of this study, as such, its ability to survive in OCN stress conditions is of great interest (Figure 4.1). Further, the rapid and effective consumption of OCN seen in *P. fluorescens* is vital (Figure 4.2). Within the first 12 hours of inoculation, overall OCN levels drop to 48.01 ± 11.84 %, and by 24 hours OCN levels had effectively reached 0 %. Biomass in OCN stress cultures also match this trend, reaching stationary phase at 24 hours (Figure 4.2). This rate is slower than control cultures, however, which only require 12 hours to reach stationary phase (Figure 4.1).

Unlike OCN, the consumption of SCN requires great deal of time, as at 96 hours of growth, 92.79 ± 0.60 % of SCN remained, but 24 hours later total remaining SCN dropped to 1.27 ± 1.79 % (Figure 4.6). Growth in SCN stress cultures require greater than 140 hours to reach stationary phase, a significant increase from both the 24 hours seen with OCN of equal concentration, and the 72 hours seen in SCN controls (Figures 4.1, 4.6. 4.5). These findings suggest that *P. fluorescens* must first adapt to the presence of SCN prior to its degradation, whereas the molecular mechanisms required for OCN degradation may be constitutively expressed. Additionally, both SCN and OCN were present at 10 mM in media, which is the highest reported concentration of SCN found in effluent [10]. Overall, these findings indicate that *P. fluorescens* is a strong cyanotroph, as both cyano-derivatives were fully metabolized from high concentrations.

While the adaptation to SCN does account for some of the time increase seen in reaching stationary phase, it does not account for all of it. The cultures for SCN studies (including control) have an initial pH ~10.00, while OCN studies have an initial pH ~7.00.
Comparing OCN and SCN control cultures, the time required to reach stationary phase increases from 24 hours to 72 hours in OCN and SCN, respectively (Figure 4.1, 4.5). This difference of 48 hours suggests that the high pH is not ideal for growth. In all cases, the cultures modified the pH to be basic, in the range of 9.00-9.50 (data not shown).

While TcDH was not detectable using BN-PAGE coupled with formazan production, cyanase was detected and was found to be active at higher levels in membrane fractions than soluble fractions (Figure 4.3). Additionally, the presence of SCN slightly increased the activity of cyanase relative to control (Figure 4.7). In OCN stress cultures, it was found that cyanase activity was actually decreased relative to control cultures (not shown). Given that the presence of OCN should require an increase in cyanase activity for effective degradation, this finding is contrary to what was expected. However, a possible explanation may be that cyanase resides in the membrane, and is stored until the presence of OCN facilitates the releases of cyanase into the extracellular matrix. Whether this is the case or if another factor is affecting activity is currently unknown. The activity of cyanase in control samples was found to be relatively high in every cyanase activity assay, in both OCN and SCN stress cultures. This finding, coupled with the rapid adaptation of *P. fluorescens* to the presence of OCN, would strongly suggest that the cyanase is constitutively expressed, allowing the bacteria to instantly respond to a new source of nitrogen.

In order to confirm the presence of cyanase in membrane fractions, membrane CFE fractions were treated with 2 mM OCN, and OCN levels were determined after 1 hour (Figure 4.5). Initially, ammonia hydrolyzed from OCN accounted for 99 % of all ammonia measured. However, after 1 hour of incubation, 73 % of all ammonia measured was free ammonia, while the hydrolyzed OCN accounted for only 27 %. This finding strongly
supports the presence of a OCN hydrolyzing enzyme, the most likely being cyanase. Cyanase, as previously stated, is responsible for degrading OCN into ammonia and bicarbonate (HCO₃⁻) (Equation 5). Since OCN was provided as the sole nitrogen source, the bacteria would have required mechanisms for the incorporation of ammonia into its metabolism. One such mechanism is likely to be the conversion of α-ketoglutarate to glutamate by GDH (Figure 5.1). In future studies, the activity of GDH (and other possible nitrogen fixing enzymes) would be monitored, as it would provide further insight into how *P. fluorescens* adapts to OCN stress. The activity of GDH could also be monitored under SCN stress, as it should require a similar mechanism, given that OCN is the presumed intermediate in SCN degradation (Figure 1.1). Further, the bacteria’s ability to metabolize HCO could be assessed, as the production of large quantities of HCO during OCN hydrolysis could provide the cell with an increase in available carbon. A likely mechanism for said integration is the production of oxaloacetate from pyruvate, catalyzed by pyruvate carboxylase; therefore, any future studies on the metabolism of OCN should examine the activity of pyruvate carboxylase, and any other carboxylases typically expressed at high levels, as they are likely to play a key role in the complete integration of OCN into metabolism (Figure 5.1).

In an attempt to accelerate SCN consumption and circumvent the lag phase seen in SCN stress cultures, cultures were grown using OCN as sole nitrogen source in the presence of either 5 or 0.5 mM SO₄²⁻, then supplemented with 10 mM SCN after reaching stationary phase (Figure 4.8). As seen in Figure 4.8, the overall concentration of SCN in the 5 mM SO₄²⁻ culture did not change over 48 hours; however, the 0.5 mM SO₄ shows a decrease of approximately 0.5 mM SCN (Figure 4.8). This finding suggests that the high concentration
**Figure 5:1 Proposed Mechanism of Metabolic Integration of Cyanate in *P. fluorescens***

Possible mechanisms for integration of mineralized OCN within *P. fluorescens*. Enzymes are denoted in red, cyanate (and mineralized OCN) is shown in grey, endogenous metabolites are shown in orange, and integrated OCN is shown in green.

Of sulphur available to the bacteria inhibited *P. fluorescens’* ability to adapt to SCN stress, even after reaching a high level of culture growth. Given the relatively low decrease in SCN levels, cultures grown using 0.1, 0.05, or even lower concentrations of SO_4^2− should be conducted to further examine this finding. However, previous research has indicated that the presence of additional sulphur species greatly inhibits the cell’s ability to degrade SCN, as the equilibrium for the reaction greatly favours the reactants (Equation 4) [12]. The high levels of SO_4^2− may decrease *P. fluorescens’* ability to mineralize SCN through various mechanisms. It may be the case that an excess of SO_4^2− in media prevents the reaction that converts SCN into SO_4^2−. Alternatively, SO_4^2− could be responsible for directly inhibiting activity or even expression of TcDH. Given that *P. fluorescens* grown in SCN stress required several days of adaptation prior to SCN mineralization, it is possible that TcDH is only
expressed in low or $\text{SO}_4^{2-}$-free environments in order to acquire new sources of sulphur. Regardless of mechanism, the destruction of SCN in effluent may be inhibited due to the presence of high concentrations of sulphate often present in mining waste waters [12]. When applied in industry, this may require pretreatment to remove the sulphates or to convert SCN into CN/OCN, or may simply require an excess of nutrients to allow \textit{P. fluorescens} to entirely degrade both sulphate and SCN. Future studies examining the expression levels of both SCNase and TcDH in low and high sulphur conditions could provide further insight to this matter, and fully elucidate the mechanism of action used by \textit{P. fluorescens}.

\textit{P. fluorescens} showed great ability to degrade OCN in the presence of less favourable carbon sources, including acetate and glycerol (data not shown). While limited in its scope, the exploratory study showed similarly rapid growth to that of the more expensive carbon source used, citrate. Of particular interest is the rapid bacterial growth seen in glycerol, which exceeded the growth of the citrate cultures. Given sufficient time, this study would ideally have examined the use of pure glycerol, and later glycerol waste from bio-fuel production. If growth using biofuel waste is sufficient, its use as a cheap and effective carbon source could be implemented in applied bioremediation.

One limitation of this study is that it does not simulate effluent which has been supplemented with minerals and nutrients, but rather simulates growth under ‘ideal’ stress conditions. A better understanding of \textit{P. fluorescens}’ ability as an effective cyanotroph in bioremediation could be determined by more accurately simulating effluent conditions. Such cases would likely include the presence of CN and metal-CN complexes, higher and potentially toxic levels of heavy metals, and in certain cases, the presence of toxic hydrocarbons, such as phenols [9,12,38]. Many of the compounds seen in effluents would
likely inhibit *P. fluorescens* growth, and could prevent effective decontamination. However, if the bacteria are able to tolerate or even consume these toxic substances, the effectiveness of its use in bioremediation would increase significantly.

The substantial amount of time required to degrade SCN has previously been reported in *P. aeruginosa*, requiring greater than 100 hours to degrade 78 % of SCN in media [27]. In order to circumvent this, and significantly increase degradation rate, CN was added to the SCN cultures, which drastically improved consumption. Under similar circumstances, *P. fluorescens* is likely to react in a similar manner, allowing for rapid and complete degradation of SCN. Given the drastic increase of SCN consumption seen in the presence of CN, it is likely that the ‘ideal laboratory conditions’ seen in this study actually represent slower decontamination of cyano-derivatives, relative to effluent-simulating media or even genuine effluent. Given that the effluent will contain some level of CN, it is likely that the same “metabolic shock” seen by Mekuto et al. would be encountered, leading to a significant decrease in decontamination time [27]. Further, a replication of this study should be conducted where the effects of CN on OCN degradation could also be monitored.
Chapter 6 Conclusion

*P. fluorescens* is readily capable of metabolizing OCN and using the freed ammonia as its sole source of nitrogen. Further, it is capable of metabolizing SCN as sole source of nitrogen and sulphur, though over a much greater period of time. The enzymatic mechanisms required for OCN degradation are likely constitutively expressed, while those used by SCN require an external signal to facilitate expression. Given that SCN consumption did not occur in presence of free sulphate, it is likely that the presence of additional sulphur sources inhibits SCN degradation, and possibly even TcDH expression. *P. fluorescens* is able to degrade OCN via cyanase, which was found to have a greater level of activity in membrane fractions. Complete degradation of both cyano-derivatives at relatively high concentrations was observed, and with further optimization the time required to achieve complete degradation could be reduced. *P. fluorescens* is a strong cyanotroph capable of readily degrading both OCN and SCN under nitrogen and sulphur free conditions, and shows promise in its potential use within industrialized bioremediation.
Chapter 7 References

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