**BIOREMEDIATION OF GOLD MINE WASTEWATER USING *FUSARIUM OXYSPORUM***

**by**

**ENOCH AKINBIYI AKINPELU**

Thesis submitted in fulfilment of the requirements for the degree
**Magister Technologiae: Chemical Engineering**

in the Faculty of
**Engineering**

at the
**Cape Peninsula University of Technology**

**Supervisor:  Prof. S.K.O. Ntwampe**
**Co-supervisor: Prof. T. V. Ojumu**

**Cape Town**
**November 2014**

# DECLARATION

I, Enoch Akinbiyi Akinpelu, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.


20/November/2014

_____                    _____
**Signature**                                                      **Date**

Cape Peninsula
University of Technology

# ABSTRACT

The legislative requirements for handling cyanide containing wastewater have become stringent internationally. Cyanide properties make it indispensable in the mining industry especially for gold recovery. The resultant wastewater generated is discarded to tailing ponds. Any leakages or total collapse of tailing ponds can result in the contamination of surface water bodies; endangering aquatic organisms' and humans' alike. The over reliance on physical and/or chemical treatment methods for cyanide wastewater treatment is not sustainable due to high input costs and the generation of by-products. A feasible alternative treatment method for cyanide contaminated wastewater is the biodegradation method, as a wide range of microorganisms can degrade cyanide. In this study, the cyanide biodegradation ability of *Fusarium oxysporum* was assessed in two stages. Firstly, optimal operating conditions for maximum cyanide biodegradation were determined using a central composite design (CCD) at an elevated cyanide concentration, i.e. 500 mg F-CN/L. Thereafter, using the optimum conditions obtained, (i.e temperature 22°C and pH 11), cyanide biodegradation kinetics and microbial growth kinetics in the cultures at lower cyanide concentrations of 100, 200 and 300 mg F-CN/L were assessed. This was followed by the assessment of cyanide biodegradation at a temperature of 5°C, which was used to simulate winter conditions. In general, lower cyanide concentrations are used in the extraction of gold, therefore, the resultant wastewater will contain free cyanide concentration less than 300 mg F-CN/L.

For the first stage of experiments, an isolate, *Fusarium oxysporum* from cyanide containing pesticides was cultured on potato dextrose agar (PDA) plates, followed by incubation at 25°C for 5 days. A response surface methodology (RSM) was used to evaluate design parameters for the biodegradation of cyanide by this fungus. The temperature evaluated at this stage ranged from 9°C to 30°C and pH range of 6 to 11 in cultures solely supplemented with agrowaste, i.e *Beta vulgaris* waste. *Beta vulgaris* is commonly known as Beetroot. The *Fusarium oxysporum* inoculum (2% v/v) was grown on a *Beta vulgaris* waste solution (20% v/v), as the sole carbon source in a synthetic gold mine wastewater (39% v/v) containing heavy metals; arsenic (7.1 mg/L), iron (4.5 mg/L), copper (8 mg/L), lead (0.2 mg/L) and zinc (0.2 mg/L), for 48 hours using a rotary shaker at 70 rpm. Thereafter, free cyanide as a potassium cyanide solution (39% v/v), was added to the cultures to make a final cyanide concentration of 500 mg F-CN/L in the culture medium which was incubated for a further 72 hours at 70 rpm. Optimal operating conditions for the biodegradation of cyanide were then determined using a numerical option in the Design-Expert® software version 6.0.8 (Stat-Ease Inc., USA).

iii

Subsequently, using the optimal pH obtained (pH =11) and a preselected temperature of 5°C (to represent winter conditions), cyanide biodegradation rates and microbial growth kinetic studies were carried out using *Beta vulgaris* waste containing a *Fusarium oxysporum* (0.7% v/v; grown overnight) inoculum in wastewater (32.7% v/v) and potassium cyanide in phosphate buffer (53.7% v/v). The cultures contained 100, 200 and 300 mg F-CN/L. The cultures were incubated in an orbital shaker at 70 rpm for 144 hours and samples taking every 24 hours. An Ordinary Differential Equation (ODE) solver (Polymath) was used for modelling cyanide degradation kinetics while the Monod's growth kinetic model was used to monitor the microbial growth parameters of the cultures.

For the first stage, the optimum operating conditions were determined as a temperature of 22°C and a pH of 11 for maximum cyanide biodegradation of 277 mg F-CN/L from an initial cyanide concentration of 500 mg F-CN/L over a 72 hour period, with residual ammonium-nitrogen and nitrate-nitrogen of 150 mg $NH_4^+$-N/L and 37 mg $NO_3^-$-N/L, respectively. Although, the residual ammonium-nitrogen inhibited cyanide biodegradation, it was consumed as a nitrogen source for microbial growth. The *Beta vulgaris* waste was determined to be a suitable substrate for cyanide degradation.

From the biodegradation response quadratic model, temperature was determined to influence cyanide biodegradation. For the cyanide degradation kinetics, at an optimum temperature of 22°C, the biodegradation efficiency was 77%, 58% and 62% with the corresponding maximum microbial population of 1.56 x $10^7$, 1.55 x $10^7$ and 1.57 x $10^7$ CFU/mL for 100, 200 and 300 mg F-CN/L, being achieved. An indication that the *F. oxysporum* cultures were efficient at lower cyanide concentration. Furthermore, at a temperature of 5°C, the biodegradation efficiency, although slightly lower, was 51%, 43% and 44% with the corresponding maximum microbial population of 1.21 x$10^7$, 1.11 x $10^7$ and 1.12 x $10^7$ CFU/mL for 100, 200 and 300 mg F-CN/L cultures, respectively, with minimal differences observed for cultures with 200 and 300 mg F-CN/L. The cyanide biodegradation rates increased with temperature increases and varied with different cyanide concentrations below 500 mg F-CN/L. The estimated energy of activation for cyanide degradation for a change in temperature from 5°C to 22°C using the Arrhenius model was 19.6, 12.7 and 14.9 kJ/mol for 100, 200 and 300 mg F-CN/L, respectively. The means and standard deviations for rate of degradation of cyanide at 5°C and 22°C for the ODE models was 0.0052 (± 0.0011) $h^{-1}$ and 0.0084 (± 0.0027) $h^{-1}$, respectively.

Cape Peninsula
University of Technology

The inhibitory effect of the cyanide was quantitatively pronounced under cold temperature as the heavy metals, residual ammonium-nitrogen and nitrate-nitrogen hindered the cyanide degradation. Similarly, microbial growth rates increased with a temperature rise (from 5°C to 22°C), resulting with a reduction in the microbial populations' doubling time. When compared with the simulated winter conditions, the specific population growth rate increased 4-fold, 5-fold and 6-fold in 100, 200 and 300 mg F-CN/L, respectively, for higher temperatures; an indication that the *Fusarium oxysporum* isolate prefers higher temperature. The estimated energy of activation for cellular respiration was 44.9, 54 and 63.5 kJ/mol for 100, 200 and 300 mg F-CN/L cultures, respectively, for the change in temperature from 5°C to 22°C. The means and standard deviations of microbial growth rate at 5°C and 22°C were: 0.0033 (± 0.0013) $h^{-1}$ and 0.0151 (± 0.0027) $h^{-1}$, respectively. The difference in error (standard deviation) of the cyanide biodegradation rate and microbial growth rate was insignificant (0.02% at 5°C) especially at temperature 22°C where there were minimal differences, indicating the reliability and reproducibility of this biodegradation system in batch operated bioreactors.

Cape Peninsula
University of Technology

# ACKNOWLEDGEMENTS

I wish to thank:

- ❖ God for giving me this opportunity when I least expected it. To him alone be all the glory,
- ❖ My supervisors, Prof. S. K. O. Ntwampe and Prof. T. V. Ojumu for their assistance, guidance and technical input throughout the duration of this study,
- ❖ My wife, for her love, support and holding forth in my absence,
- ❖ My parents and the entire Akinpelu family, for their encouragement and support to make this vision a reality,
- ❖ My friends at home and abroad, who have contributed in different ways to this success,
- ❖ Staff and fellow students of the Bioresource Engineering Research Group (BioERG) for their support, particularly Dr. Nchu and Ms N. Mpongwana, who initiated the *Fusarium oxysporum* study,
- ❖ Cape Peninsula University of Technology, University Research Fund (URF) for the financial support, without which this project would not have been possible.

Cape Peninsula
University of Technology

# DEDICATION

To

THE BEGINNING OF MY STRENGTH, **DAVID**

AND

THE EVIDENCE OF GOD'S PRESENCE, **EMMANUEL**

Cape Peninsula
University of Technology

# RESEARCH OUTPUTS

The following outputs are contributions made by the candidate to scientific knowledge and development during his masters' candidacy (2014):

❖ **Posters**

**Akinpelu EA**, Ntwampe SKO, Ojumu TV. 2014. Potential of *Fusarium oxysporum* in the biodegradation of free cyanide containing wastewater under cold temperature conditions using agro-waste. The Southern African Institute of Mining and Metallurgy Western Cape Branch Conference, 7 - 8 August. Cape Town, South Africa.

❖ **Presentations**

**Akinpelu EA**, Ntwampe SKO, Ojumu TV. 2014. Biodegradation of free cyanide containing wastewater using *Fusarium oxysporum* supplemented with *Beta vulgaris* agro-waste. 2$^{nd}$ International Conference U6 Consortium, 5 - 10 September. Cape Town, South Africa.

**Akinpelu EA**, Ntwampe SKO, Ojumu TV. 2014. *Beta vulgaris* augumented nitrification in free cyanide wastewater under cold temperature using *Fusarium oxysporum*. Cape Peninsula University of Technology Postgraduate Conference, 5 November. Cape Town, South Africa.

❖ **Publications**

**Akinpelu EA**, Amodu OS, Mpogwana N, Ntwampe SKO, Ojumu TV. 2014. Utilisation of agrowaste in biodegradation of cyanide contaminated wastewater. *Biotechnology, ISSN:978-953-51-41570, D. Ekinci, Ed. Croatia: InTech*: 1-19

**EA Akinpelu,** S.K.O. Ntwampe, N. Mpongwana, F. Nchu, T. Ojumu. Biodegradation kinetics of free cyanide in *Fusarium oxysporum-Beta vulgaris* waste-metal (As, Cu, Fe, Pb, Zn) cultures under alkaline conditions – *submitted to BioResources in 2015* (*reference* BioRes 7157)

# TABLE OF CONTENTS

Cape Peninsula
University of Technology

Cape Peninsula
University of Technology

# LIST OF FIGURES

Cape Peninsula
University of Technology

# LIST OF TABLES

# LIST OF SYMBOLS

**Nomenclature**

| Symbol | Description | Units |
|---|---|---|
| $\alpha_o$ | Constant | _ |
| $\alpha_i$ | Linear coefficient | _ |
| $\alpha_{ii}$ | Quadratic coefficient | _ |
| $\alpha_{ij}$ | Interactive coefficient | _ |
| A | Temperature | °C |
| B | pH | _ |
| $X_i$ | Coded independent variables | Undefined |
| $Y$ | Response variable | Undefined |
| $CN_B^-$ | Biodegraded cyanide | mg/L |
| $CN_I^-$ | Initial cyanide | mg/L |
| $CN_R^-$ | Residual cyanide | mg/L |
| $CN_V^-$ | Volatilised cyanide | mg/L |
| $CN_{IC}^-$ | Initial cyanide in the control | mg/L |
| $CN_{FC}^-$ | Final cyanide in the control | mg/L |
| $\mu$ | Specific growth rate constant | $h^{-1}$ |
| $t$ | Time | h |
| $K$ | Reaction rate constant | $h^{-1}$ |
| $E$ | Activation energy | KJ/mol |
| $\varepsilon$ | Error | _ |

# GLOSSARY

| Abbreviation | Description |
| --- | --- |
| ATP | Adenonine triphosphate |
| *B. vulgaris* | *Beta vulgaris* |
| CCD | Central composite design |
| COD | Chemical oxygen demand |
| F-CN | Free cyanide |
| HCN | Hydrogen cyanide |
| KCN | Potassium cyanide |
| $NH_4^+$-N | Ammonium-nitrogen |
| $NO_3^-$-N | Nitrate-nitrogen |
| ODE | Ordinary differential equation |
| RSM | Response surface methodology |
| SAD-CN | Strong acid dissociable cyanide |
| SPSS | Statistical package for the social sciences |
| WAD-CN | Weak acid dissociable cyanide |

Cape Peninsula
University of Technology

# CHAPTER 1

# INTRODUCTION

# CHAPTER 1
# INTRODUCTION

## 1.1 Background

Industrialization and the extraction of natural resources have resulted in large-scale environmental contamination and pollution. Large quantities of toxic waste have been discharged to contaminated sites which are spread across the world especially in developing countries where legislative requirements regarding environmental issues is neglected. Thus, a large number of the world's population is being exposed to contamination from past and present industrial activities, including emissions to natural resources (i.e. air, water and soil) even in the most remote regions. The danger to human and ecological system is prominent and there are observations that this assemblage of pollutants contributes in no small measure to the global epidemic of cancer and other degenerative diseases (Dubey *et al.,* 1995; Maniyam *et al.*, 2011).

Cyanide is a carbon-nitrogen radical, available in a large variety of forms and its presence in the environment is attributed to the sources related to various industrial activities. Cyanide is often found as a pollutant in wastewater through its release from metal finishing, electroplating, gold processing, coal coking and many other large scale processes, which are some of the main sources that account for the bulk occurrence of cyanide in the environment ((Dash *et al.*, 2009b)). Gold mine wastewater generally contains heavy metal pollutants such as copper (Cu), arsenic (As), iron (Fe), zinc (Zn), lead (Pb) including cyanide complexes at elevated concentrations (33.2 - 100.2 mg F-CN/L) (Shukla *et al.*, 2005; Acheampong *et al.*, 2010), because cyanide readily binds with gold, silver and other metals (Zagury *et al.*, 2004).

Cyanide in various forms is highly toxic, carcinogenic and mutagenic especially at high concentration (Maniyam *et al.*, 2011). Inorganic cyanide and nitrogenous compounds are distributed widely in the environment, mostly as a result of anthropogenic activities but also through cyanide synthesis by a range of organisms including higher plants, fungi and bacteria which produce minute concentrations of these compounds. The major source of cyanide in soil and water is through the discharge of wastewater containing a variety of inorganic cyanides and other nitrogen-based compounds (Dubey *et al.*, 1995). Cyanide is often found in organic, hydrocarbons or as an inorganic, transition, alkali and alkali earth metal complex. Many cyanide

2

complexes are highly unstable, thus temperature, pH and light can degrade the components to form free cyanide which is the most toxic form of cyanide (Rao *et al.*, 2010). Currently, the release of cyanide worldwide is estimated to be > 14 million kg per annum (Ntwampe *et al.*, 2013).

Nowadays, it is very popular to use chemical treatment methods for the conversion or oxidation of free cyanide and cyanide complexes compared to biotechnological treatment methods. Chemical remediation methods such as hydrogen peroxide oxidation are currently the most widely used methods in many operations for the treatment of cyanide contaminated wastewater. However, hydrogen peroxide oxidation is limited to the treatment of effluent, and thus cannot be used for the treatment of tailing slurries, of which ammonium-nitrogen produced is not removed. This includes nitrates formed which can then accumulate thus polluting surface waters. Chemical oxidation is particularly ineffective in the treatment of cyanide-metal complexes containing heavy metals, such as copper, nickel and silver, due to the slow reaction rates achievable (Patil *et al.*, 2000). The high quantity of hydrogen peroxide used in the treatment process increases the chemical oxygen demand (COD) of the wastewater thereby rendering the water undesirable for reuse, toxic to aquatic life as it may contain other organic substances (Dubey *et al.*, 1995).

Likewise, other oxidation methods are unsuitable due to high capital cost, specialist equipment, maintenance requirements, unfavourable by-product formation and the reduced ability to treat metal-cyanide complexes; which can result in ecological degradation (Maniyam *et al.*, 2011; Santos *et al.*, 2013a). Biological cyanide degradation is increasingly becoming the most acceptable and feasible process in industry with an assortment of research studies being conducted in this niche area (Hubbe *et al.*, 2011). Several microorganisms have been found to be effective for the biodegradation of cyanide in wastewater (Dash *et al.*, 2009b). The microbial species can convert both free cyanide and cyanide complexes to ammonium-nitrogen which can serve as a nitrogen source to the microorganism. However, the cost of refined nutrients for sustenance of these microorganisms is high hence the need for an alternative substrate, such as agrowaste.

Cape Peninsula
University of Technology

One agricultural substrate (waste) of interest is *Beta vulgaris* waste, which was shown to increase the degradation of cyanide, although not as efficiently as other agricultural waste as shown in the study of Ntwampe and Santos (2013). Furthermore, optimization of such a process may enhance the efficacy of biodegradation rates and contribute to effective bioprocess implementation on a large scale and circumvent limitations associated with the use of other refined substrates.

For large scale operations treating cyanide wastewater, the use of a robust design can be implemented if designed in a manner which can suit microbial cultivation. However, a suitable substrate, such as agricultural waste, is needed to support microbial activity when such a bioreactor is in operation. Furthermore, there is limited information on the extended use of agricultural waste for cyanide degradation without the use of refined sugars for bio-augmentation. In designing such a system, microbial activity including biofilm formation can be easily controlled as dead biomass can be sloughed-off maintaining a biofilm community that can be sustained for a longer period while the dead biomass can serve as a carrier for living cells and the biosorption of heavy metals (Coello *et al*., 2010; Enayathali *et al*., 2012).

## 1.2 Problem statement

Some of the biological processes for the detoxification of cyanide have focused on cyanide-degrading fungi with several studies establishing sustainable processes using bacterial strains such as *Klebsiella sp., Pseudomonas sp.*, and *Bacillus sp.* Both free cyanide and metal-cyanide complexes can be biodegraded to ammonium-nitrogen and further into nitrates to produce harmless nitrogen gas by suitably designed bioremediation processes. The challenge is to develop an innovative and a cost-effective process for the treatment of cyanide containing wastewater before it is discharged into the environment, and to make the water recoverable for reuse, with a primary objective of protecting the environment and the ecosystem supporting life. It is proposed that a suitable bioreactor system be designed to treat gold mining wastewater containing cyanide, including metals (arsenic, copper, lead, iron, and zinc) to an acceptable standard for discharge to the environment or re-use, in a system solely bioaugmented with agro-waste.

Cape Peninsula
University of Technology

**1.3 Hypothesis**

It is hypothesized that a biodegradation system utilising agricultural waste, *Beta vulgaris* and *Fusarium oxysporum*, can efficiently support the biodegradation of cyanide in gold mine wastewater for safe discharge/reuse by the mining industry.

**1.4 Research objectives**

The research objectives were:

- to assess the suitability of *Beta vulgaris* waste with *Fusarium oxysporum* for the effective biodegradation of cyanide containing gold mine wastewater;
- to optimise physico-chemical conditions for cyanide biodegradation;
- to analyse the effect of temperature on cyanide biodegradation and the microbial growth at optimum cyanide degradation conditions;
- to monitor the microbial growth during cyanide degradation;
- to evaluate the activation energy for cyanide degradation and cellular respiration;
- to develop a model for cyanide biodegradation kinetics and microbial growth; and
- to quantify ammonium-nitrogen and nitrate formation during cyanide degradation.

**1.5 Significance of study**

This study developed a model for biodegradation of cyanide in gold mine wastewater. In this process the suitability of *Beta vulgaris* waste as the sole nutritional source to support the microbial activity of *Fusarium oxysporum* for cyanide degradation was assessed, which in turn can reduce costs for the mining industry in South Africa.

**1.6 Delineation of study**

The following were not considered in this study:

- Scale up of experiments to a large scale;
- Economic evaluation and feasibility studies;
- Enzyme identification and activity;

Cape Peninsula
University of Technology

- Biosorption of heavy metals was not quantified although their concentration in the wastewater used was known at the beginning of each experiment, i.e. the sole focus of the study was the degradation of cyanide in the presence of arsenic, copper, iron, lead and zinc for a process supported with *Beta vulgaris* waste.

Cape Peninsula
University of Technology

# CHAPTER 2

# LITERATURE REVIEW

# CHAPTER 2
# LITERATURE REVIEW

## 2.1 Introduction

This chapter explored the challenges associated with the remediation of cyanides and its complexes in wastewater. It also discusses the available treatment methods, application of agricultural waste in cyanide microbial degradation processes and current developments that give an indication on the suitability of microbial systems for the bioremediation of cyanide compounds under varying environmental conditions.

## 2.2 Gold Mining Operations

Gold-bearing ore is pulverized in a multistage crushing and milling circuit. The ores can either be readily available or locked up within a sulphide mineral before recovery by a cyanidation process. The ore is ground to produce high-grade sulphide containing concentrate which is then oxidized. This liberates the gold particles and makes them available to recover by the cyanidation process. The ores are then processed by leaching the ore in a cyanide solution for about 16 to 48 hours followed by the adsorption of the gold cyanide complex onto activated carbon. The wastewater generated is discharged into tailing ponds. The gold containing activated carbon is then rinsed before the gold is separated from the carbon using a high pH solution containing caustic soda and cyanide. The stoichiometric equation describing the process is as shown in Equation (2.1). The gold from the eluate is then deposited onto the cathode of electrowinning cells which is followed by smelting into ingots that can be shipped to gold refineries. Figure 2.1 shows a diagrammatic representation of the cyanidation process.

$$2Au + 4NaCN + 2H_2O + O_2 \rightarrow 2NaAu(CN)_2 + 2NaOH + H_2O_2 \qquad (Eq. 2.1)$$
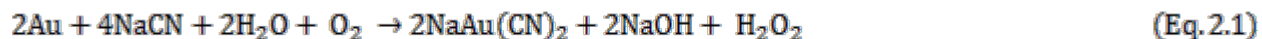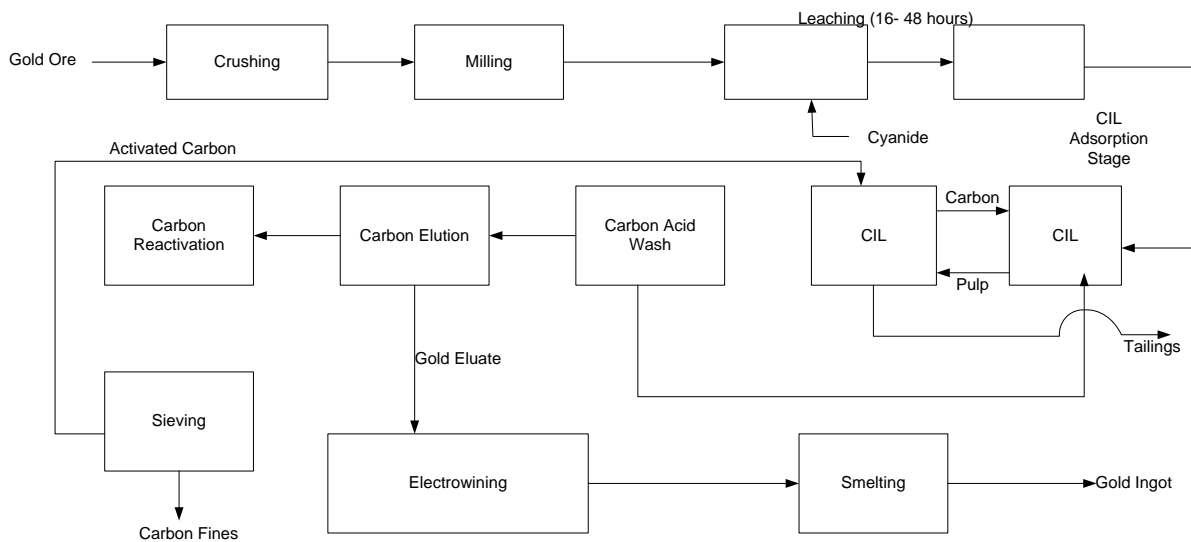
**Figure 2.1: Diagrammatic representation of the cyanidation process of gold extraction (Acheampong *et al.*, 2010)**

### 2.2.1 Environmental risk of cyanide and heavy metals

Cyanides are a group of compounds that contain a carbon and nitrogen atom joined with a triple bond. They are available in several forms including; hydrogen cyanide; simple inorganic salts such as sodium cyanide (NaCN) and potassium cyanide (KCN); complex metal cyanides, such as thiocyanates (SCN), and as nitriles where the cyanide binds to an organic radical. The toxicity of cyanide and environmental concerns from its continued industrial use continue to generate interest (Ma *et al.*, 2010).

Similarly, gold processing operations contain heavy metals even at low concentrations. These heavy metals include lead (Pb), copper (Cu) and arsenic (As) which are toxic to both plants and animals, constituting a risk to the ecosystem due to their bioaccumulation properties in living tissue (Acheampong *et al.*, 2013). Several environmental hazards are associated with heavy metals and cyanide in wastewater stored in tailing ponds due to the risk of failure of the ponds. A case study of such hazards was in the year 2000, when a tailing storage facility in northern Romania collapsed, whereby metallic-cyanide containing wastewater ($1 \times 10^6$ m$^3$) and a heavy metal sludge was spilled, releasing an estimated 122 tonnes of cyanide into the river Tisza (Rico *et al.*, 2008).

9

South Africa contributes in no small measure to gold mining activities globally; as of 2013, her known gold reserves is second only to Australia, representing 11% of the world total gold reserve (see Table 2.1). South Africa's contribution to gold production in 2013 is shown in Figure 2.2. As a consequence of inherent toxicity and volume of wastewater associated with gold mining, there is a need to have an efficient and cost effective method of treating cyanide containing wastewater before discharge or reuse in order to meet discharge standards in various nations, including, South Africa.

**Table 2. 1: World Gold Reserves, 2013 (Edelstein, 2014)**

| Country | Reserve Base | | |
|---|---|---|---|
| | ton(s) | % | Rank |
| South Africa | 6000 | 11.1 | 2 |
| USA | 3000 | 5.5 | 5 |
| Australia | 9900 | 18.3 | 1 |
| Brazil | 2400 | 4.4 | 6 |
| Canada | 920 | 1.7 | 12 |
| Chile | 3900 | 7.2 | 4 |
| China | 1900 | 3.5 | 8 |
| Ghana | 2000 | 3.7 | 7 |
| Indonesia | 3000 | 5.5 | 5 |
| Mexico | 1400 | 2.6 | 10 |
| Papua New Guinea | 1200 | 2.2 | 11 |
| Peru | 1900 | 3.5 | 8 |
| Russia | 5000 | 9.2 | 3 |
| Uzbekistan | 1700 | 3.1 | 9 |
| Other | 10000 | 18.5 | - |
| Total | 54220 | | |

Cape Peninsula
University of Technology
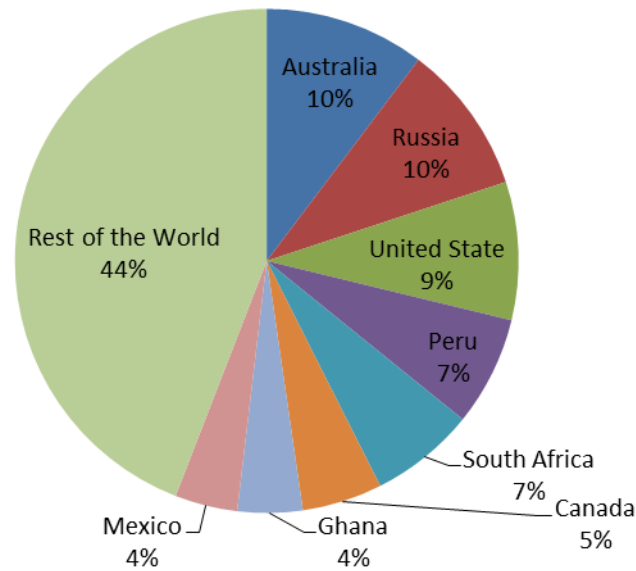
**Figure. 2.2: World Gold Production in 2013 (Rhonas *et al.*, 2014)**

## 2.3. Generation of agricultural wastes

Agricultural waste of about 1.6 billion tonnes is generated globally from food wastage (Gustavsson *et al.*, 2011). Their accumulation degenerate the environment but these wastes can be used to produce other value added products with a view of reducing production cost. These wastes can result from: (a) cereals, such as rice, maize, oats, barley, millet, sorghum among others; (b) Fruit and Vegetable, such as oranges, lemon, tomatoes, onion, apple, banana, garlic among others; (c) Roots and Tubers, such as yam, potatoes, cassava, sweet potatoes, carrots among others; (d) Oilseed and Pulses, such as shelled groundnuts, mustard seed, soybeans, lentil, sunflower among others. The waste generated was classified by Gustavsson *et al.* (2011)-see Table 2.2.

Cape Peninsula
University of Technology

**Table 2. 2: Classification of agricultural waste (Gustavsson *et al.*, 2011)**

| Waste Classification | Definition |
| --- | --- |
| Agricultural Production | Accounts for losses during mechanical handling and harvest operations. |
| Postharvest | Losses due to storage, transportation and distribution of farm produce. |
| Processing | Include losses during industrial and domestic processing. |
| Distribution | Losses in the marketing level. |
| Consumption | All household waste and losses. |

According to Gustavsson *et al.* (2011), losses resulting from agricultural waste in underdeveloped and developing countries is due to financial, managerial and technical limitations in harvesting techniques, storage and cooling facilities, packaging and marketing systems, meanwhile in developed countries it relates to consumer behaviour and the lack of coordination between different personnel in the supply chain. In sub-Saharan Africa, consumers are responsible for approximately 3.5% of total agricultural waste, with the majority being generated during the pre-consumer stages of the food chain. By contrast, in the European Union, 42% of total agricultural waste is generated by households, 39% is from the production and processing sector, 14% is contributed by the food service and catering sector, and the remaining 5% is associated with the retail/wholesale sector (Monier, 2011). Figures 2.3 represents production volume of each agricultural commodity group per region and the percentage by mass waste contribution at each food supply stage in sub-Saharan Africa. In South Africa, the estimated agricultural waste generated is 9.04 million tonnes per annum with pre-consumer waste accounting for 96% of this waste, see Figure 2.3 (Oelofse *et al.*, 2013). These quantities are large for developing economies, therefore, the waste can be used beneficially to either produce high value products and or to support low performance processes.

### 2.3.1 Characterisation of agricultural waste

Agricultural waste contains waste with cellular walls which are mainly composed of cellulose, hemicellulose and lignin; hence they are called lignocellulose materials. Cellulose is present in large quantities in such wastes. Cellulose is composed of mainly glucose units which are readily fermented by most microorganisms. There is a need to separate the cellulose from hemicellulose and lignin. Due to the recalcitrant nature of agricultural waste materials, they have

12

Cape Peninsula
University of Technology

been subjected to different treatment methods over the years to ensure the release of locked up nutrients using processes ranging from physical to chemical treatments and sometimes biological treatment. Almost all agricultural waste pre-treatment methods are milled to give a fine and decrystallised structure for increased surface area. To improve the value of agro-waste, agricultural wastes can also be subjected to steam treatment for depolymerisation and to yield easily accessible and low molecular weight substrates (Li *et al.*, 2007).

Sun and Tomkinson (2002) treated wheat straw with ultrasonic irradiation in potassium hydroxide aqueous solutions for hemicellulose hydrolysis. Similarly, *citrus sinensis* was treated with potassium hydroxide and bromobenzene after acid hydrolysis with $H_2SO_4$ prior to being used in free cyanide conversion in order to facilitate the release of hydroxyl functional groups for deprotonation of the cyanide group (Santos *et al.*, 2013b). The extra cost of chemical reagents will increase the operating cost of the process. The *Beta vulgaris* waste used in this study was dried at 80°C, milled to less than 100μm in order to increase the surface area associated with the particulate matter of the waste, hydrolysed with distilled water using a high pressure saturated steam at temperature of 116°C for 15 minutes for the release of entrapped sugars and proteins for microbial growth.

.
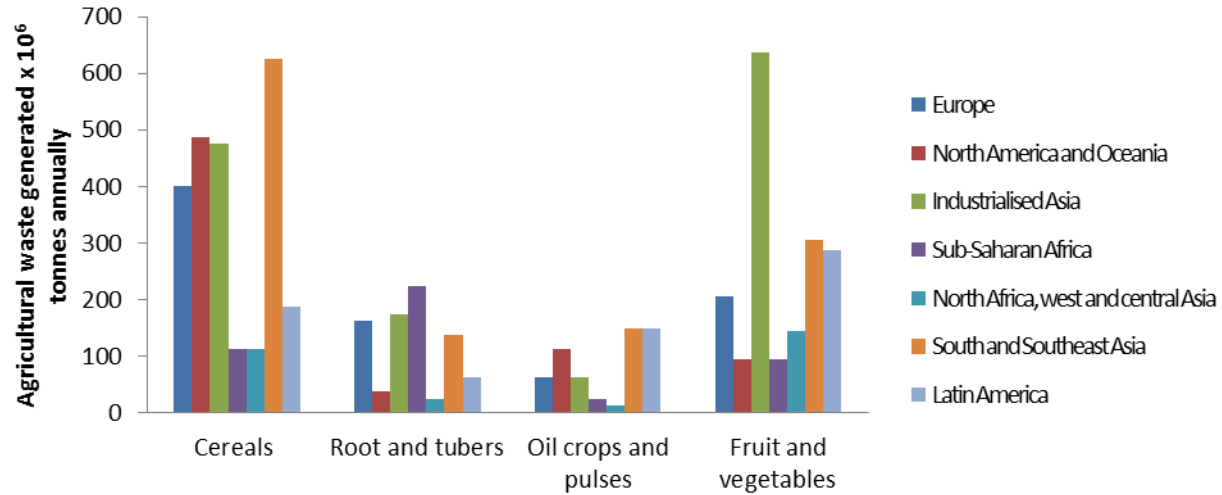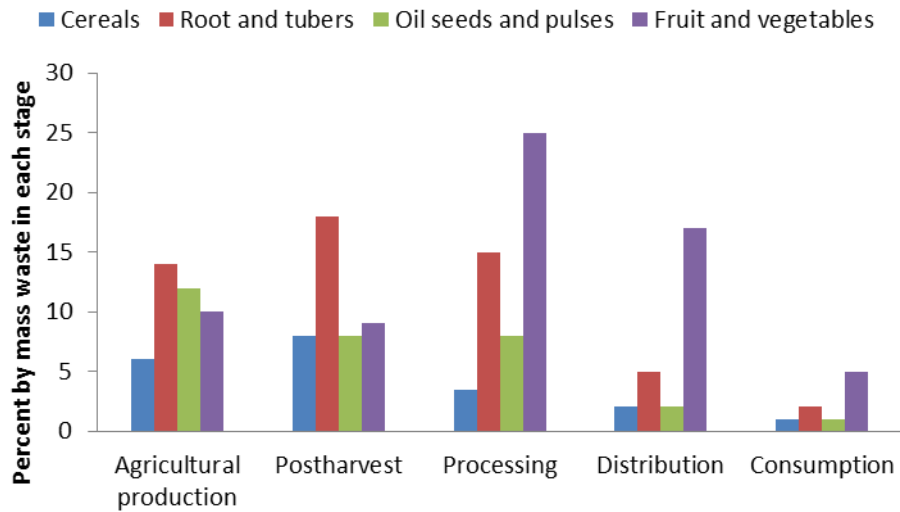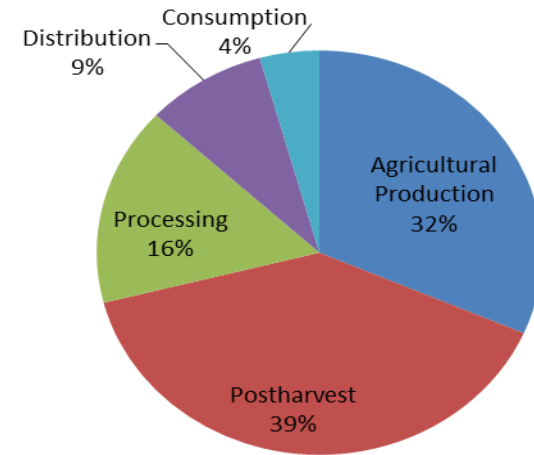
**Figure 2. 3: (a) Production volume of commodity groups (million tonnes), (b) Percent by mass waste contribution in sub-saharan Africa and (c) Percent waste contribution in South Africa (Gustavsson *et al.*, 2011; Oelofse *et al.*, 2013)**

## 2.3.2 Interaction of microbial species and agro-waste

Microorganisms use enzymes to access essential nutrients in agricultural waste. Fungi and bacterial are predominantly used for this purpose, by utilising hydrolytic and oxidative enzymes produced by these organisms to release carbon and nitrogen nutritional sources. Fungi produce enzymes for the breakdown of lignin and hemicellulose. Brown rots attacks only the cellulose while soft and white rots attack lignin and cellulose. Several strains of these fungi have been genetically modified to produce different enzymes that are capable of fermenting glucose and hemicellulose to simple sugars and then to value added products. Example of such includes; *Ceriporiopsis subvermispora*

Bacterial interactions with agro-waste involve aerobic or anaerobic systems. The bacteria produce biocatalysts for production of enzymes to breakdown lignocellulosic materials. The enzymes have oxidative and hydrolytic functions with degradation mechanisms used at different stages of degradation. One such enzyme is the cellulase group involving endoglucanase, exoglucanase and β-glucosidase enzymes which hydrolyses cellulose to simple sugars that are fermentable. Typical value added products from enzymatic agricultural waste degradation include biogas (e.g. methane), alcohols (e.g. bioethanol), and acids (e.g. acetic acid). The agro-waste undergoes anaerobic digestion involving four different stages in the production of biogas which are hydrolysis, acidogenesis, acetogenesis and methanogenesis; -see Figure 2.4 (Chattopadhyay *et al.*, 2009).

Cape Peninsula
University of Technology
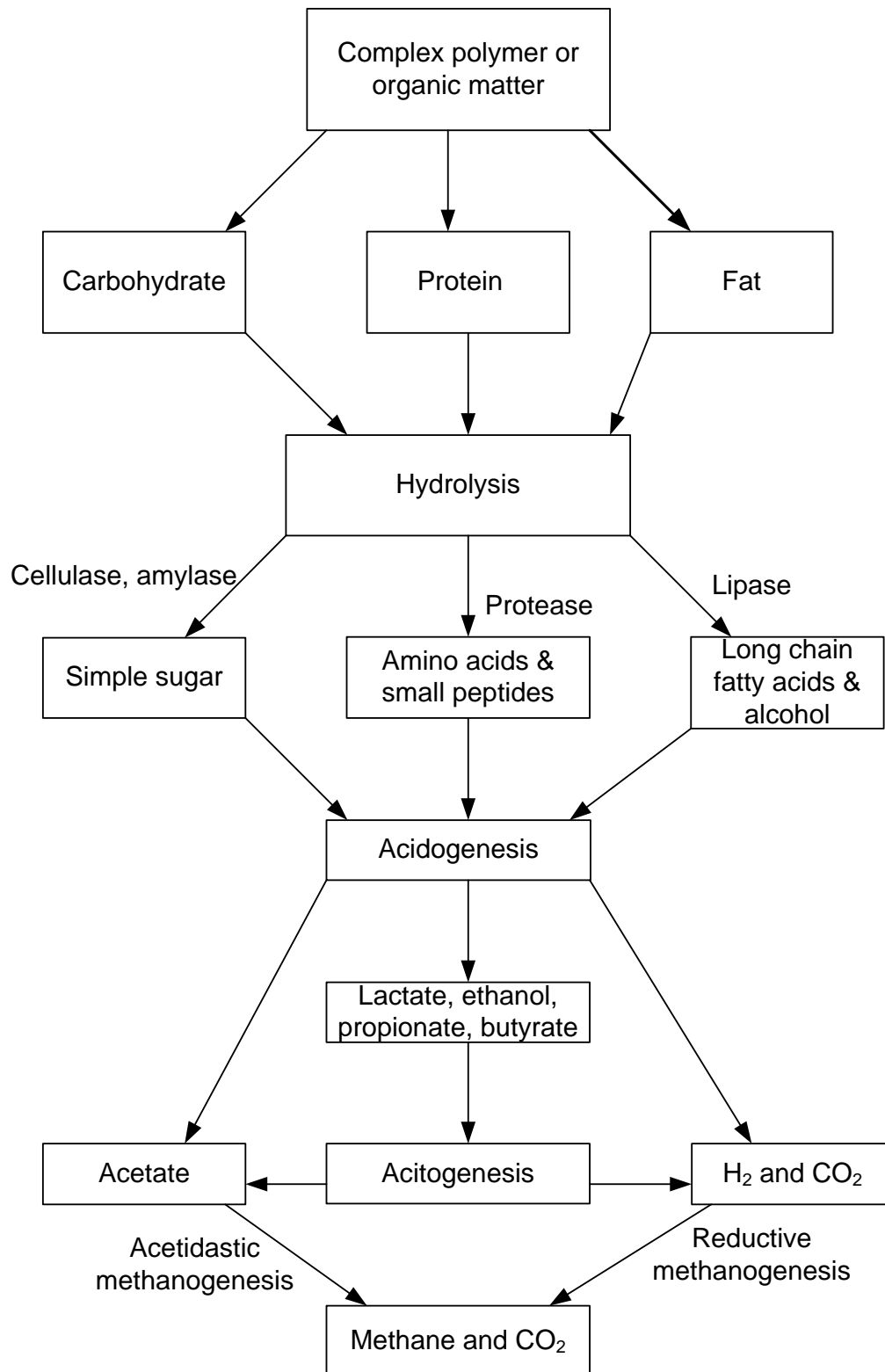
**Figure 2. 4: Anaerobic methane production phases (Chattopadhyay *et al.*, 2009).**

## 2.4 Cyanides and metallic-cyanides in gold mine wastewater

Wastewater from gold mine operations mobilise contaminants that have detrimental effects on the environment. Cyanide ($CN^-$) is a toxic chemical that is common in industrial wastewaters generated by metallurgical operations. Cyanide's strong affinity for metals makes it a favourable agent for metal finishing including treatment and as a lixivant for metal leaching, particularly gold. Gold can be dissolved in cyanide at a pH ranging from 9.5 to 10 in order to maintain an appropriate cyanide-stability for gold dissolution (Donato *et al.*, 2007). The transitional metals accompanying gold in ore, such as copper, zinc and iron, selectively bind with cyanide in the gold extraction process. Therefore, wastewater generated contain transition metals and cyanide (Donato *et al.*, 2007).

Three forms of cyanide are identified based on their solubility and stability in gold mine wastewater, namely; (a) free cyanides (F-CN) for example $CN^-$, hydrogen cyanide (HCN); (b) weak acid dissociable cyanides (WAD-CNs) such as tetracyanozincate (II) $[Zn(CN)_4]^{2-}$ and tricyanocuprate (I) $[Cu(CN)_3]^{2-}$, they breakdown into F-CN and the transition metal when in a weak acid solution (pH $\leq$4.5); (c) strong acid dissociable cyanides (SAD-CNs) are metal-cyanide complexes which are more stable than WAD-CNs and breakdown into F-CN and transition metals when in a strong acid aqueous solution (pH $\leq$1), examples are hexacyanoferrate (III) $[Fe(CN)_6]^{3-}$, hexacyanoferrate (II) $[Fe(CN)_6]^{4-}$ and tetracyanomercurate (II) $[Hg(CN)_4]^{2-}$.

In view of billions of tonnes of gold ore that are being leached every year with cyanide, there is a need to prevent surface and ground water contamination, hence, methods for the safe and suitable treatment, storage and handling of wastewater containing cyanide should be of primary concern in gold mine operations. The ability of *Fusarium oxysporum* to produce different enzymes when grown on beetroot (*Beta vulgaris*) have been identified (Anuradha *et al.*, 2010). Moreover, *Fusarium oxysporum* has been identified to endure high cyanide concentration up to 520 mg/L (Pereira *et al.*, 1996). This study explored the potential to enhance cyanide degradation in the presence of heavy metals, using *Fusarium oxysporum-Beta vulgaris* cultures.

Cape Peninsula
University of Technology

## 2.4.1 Regulatory discharge limits for wastewater containing cyanide and metals

There are several human activities that have contributed enormously to cyanide contamination in soil and water. The most pronounced is the discharge of wastewater to sewage treatment plants especially from facilities that serve industrialized areas where cyanide can reach 21.6 mg/L (Acheampong *et al.*, 2013). Despite its toxicity, the demand for cyanide in many processes such as steel manufacturing, gold mining and electroplating is significant. Various industries release a combination of free cyanide and cyanide complexes into the environment via a variety of disposal methods. As a result, regulatory bodies of different nations have set up the optimum discharge limit for release of toxic metals such as copper, arsenic, lead, iron, nickel, zinc, etc., into surface water systems. Table 2.3 shows the maximum contaminant level (MCL) values as per South Africa Department of Water Affairs (DWA), national water Act waste discharge standard guidelines.

**Table 2. 3: Wastewater discharge limit values, (DWA, 2010).**

| Variables and substances | Standards (mg/L) in South Africa |
|---|---|
| Arsenic (As) | 0.02 |
| Lead (Pb) | 0.01 |
| Mercury (Hg) | 0.005 |
| Cadmium (Cd) | 0.005 |
| Chromium (Cr(VI)) | 0.05 |
| Zinc (Zn) | 0.1 |
| Iron (Fe) | 0.3 |
| Copper (Cu) | 0.01 |
| Selenium (Se) | 0.02 |
| Cyanide (CN) | 0.02 |

## 2.4.2 Treatment methods of gold mine wastewater

Cyanide treatment and heavy metal removal from gold mine wastewater can be removed by several methods, namely; oxidation, filtration, reverse osmosis, electrodialysis, membrane electrolysis, etc. (Acheampong *et al.*, 2010). However, there are numerous short comings in the application of these methods. Free cyanide in tailing ponds can degrade to less toxic chemicals by: (a) physical; (b) chemical; and (c) biological processes. The simplest method of removing cyanide in gold mine wastewater is through natural degradation which occurs by volatilization and photodecomposition of cyanide in tailing storage facilities (TSF) although the degradation rates may vary. The rate of conversion from the liquid phase to gaseous cyanide depends on

Cape Peninsula
University of Technology

the depth of the pond and its surface area with most of the liberated cyanide occurring below pH level 8 to 9.2 in the tailing ponds (Donato *et al.*, 2007).

Similarly, metal-cyanide complexes are known to be stable, insoluble or slightly soluble in water. Compounds such as $Cu(CN)_3$ and $Zn(CN)_4$ are insoluble in water but they are soluble in ammonium solutions. They decompose slowly to liberate cyanide when exposed to ultraviolet light especially at room temperature. Overall, photo degradation is slow in deep, or turbid waters (Donato *et al.*, 2007). Other procedures for treating cyanide predominantly involve separation or destruction processes. Cyanide can be recovered for recycling through separation using physical or complexation methods. Meanwhile, to produce non-toxic or less toxic cyanide, the triple bond in carbon-nitrogen is broken through a suitable destruction process. The carbon and nitrogen in the cyanide molecule experience a change in their oxidation number during the oxidation (Young *et al.*, 1995). Further discussions on physical and chemical methods are elucidated in the subsequent section.

### 2.4.2.1 Cyanide reduction using alkaline chlorination

This method is one of the cyanide destruction processes for the treatment of free cyanides and weak acid dissociable cyanides. The chlorine gas is used under alkaline conditions transforming the cyanide to a cyanogen chloride by the reaction of the cyanide and chlorine gas (Nsimba, 2009; Santos, 2013):

$$CN^- + Cl_2 \rightarrow CNCl + Cl^- \qquad (Eq. 2.2)$$

This is followed by the rapid alkaline based hydrolysis of the cyanogen chloride to cyanate;

$$ClCN^- + 2OH^- \rightarrow OCN^- + Cl^- \qquad (Eq. 2.3)$$

During the third stage, oxidation of the cyanate by chlorine gas is achieved to form nitrogen gas and a carbonate;

Cape Peninsula
University of Technology

$$2OCN^- + 6OH^- + 3Cl_2 \rightarrow 2HCO_2^- + 6Cl^- + 4OH^- + N_2 \qquad \text{(Eq.2.4)}$$
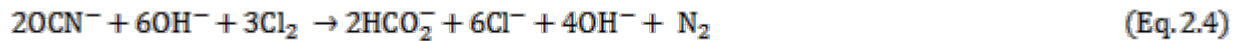
Although this process oxidises free cyanides and WAD-CN efficiently with no copper catalyst requirements, it is however, not selective, causing high consumption of reagents and may leave chlorine in solution and halomethanes which are undesirable.

### 2.4.2.2 Cyanide reduction using the INCO Sulphur dioxide/air

Sulphur dioxide/air process was first patented by the International Nickel Company (INCO). It is a destructive process involving the oxidation of free and WAD-CN using a gaseous mixture of sulphur dioxide and oxygen derived from air, in the presence of a soluble copper catalyst, as shown in Equation 2.5 and 2.6, respectively (Devuyst *et al.*, 1989). The reaction usually proceeds at pH levels 8 to 9 but there would be a decrease in the performance whenever the pH fluctuates.

$$CN^- + SO_2 + O_2 + H_2O \xrightarrow{cu} OCN^- + H_2SO_4 \qquad \text{(Eq.2.5)}$$

$$M(CN)_4^{-2} + 4SO_2 + 4O_2 + 4H_2O \xrightarrow{cu} M(OH) + 4OCN^- + 4H_2SO_4 \qquad \text{(Eq.2.6)}$$
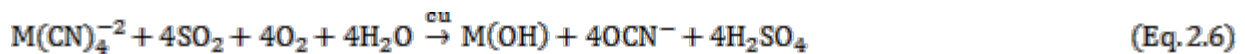
The thiocyanate produced requires further processing to oxidise it which results in increased reagent usage and thus operating cost. However, the cyanide complexes can be reduced to another state, (Equation 2.7), followed by precipitation as an insoluble metal, e.g. ferrocyanide, (Equation 2.8) (Santos, 2013):

$$2M(CN)_6^{-3} + SO_2 + 2H_2O \xrightarrow{cu} 2M(CN)_6^{-4} + 4H^+ + SO_4 \qquad \text{(Eq.2.7)}$$

$$2M^{+2} + Fe(CN)_6^{-4} + 2H_2O \xrightarrow{cu} M_2Fe(CN)_6 . 2H_2O \downarrow \qquad \text{(Eq.2.8)}$$
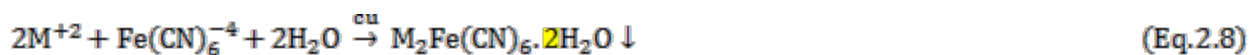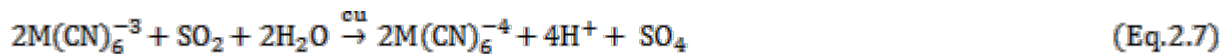
Where M stands for alkali metals.

The process is known to yield low metallocyanide effluent concentrations. It is efficient in batch and continuous systems but cyanide cannot be recovered. If the cyanide concentration is high, reagent and power costs also increase.

### 2.4.2.3 Cyanide reduction using hydrogen peroxide

The reaction involves the oxidation of free cyanides and WAD-CN in the presence of soluble copper to increase the rate of reaction as shown in Equations 2.9, 2.10 and 2.11, respectively (Khodadadi *et al.*, 2005):

$$CN^- + H_2O_2 \xrightarrow{cu} CNO^- + H_2O \qquad\qquad (Eq.\,2.9)$$

$$M(CN)_4^{-2} + 4H_2O_2 \xrightarrow{cu} M(OH)_2 \downarrow + 4OCN^- + 4H_2O \qquad\qquad (Eq.\,2.10)$$

$$OCN^- + 2H_2O \xrightarrow{cu} CO_3^- + NH_4^+ \qquad\qquad (Eq.\,2.11)$$
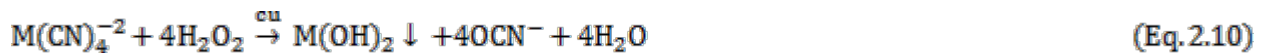
Where M stands for alkali metals.

The hydrogen peroxide process operation's design although simple, requires similar capital costs comparable with other chemical processes. Furthermore, generated sludge, ammonium or thiocyanate are not removed and the tailing slurries cannot be treated.

### 2.4.2.4 Cyanide recovery using ion-exchange

Ion exchange is a process that uses anion or cation exchange resin for adsorption metals and cyanides from wastewater. This treatment method recovers metals easily, produces minimal secondary pollutants and the disposal of regeneration agents is still a challenge (Kim *et al.*, 2002) .

Cape Peninsula
University of Technology

**2.4.2.5 Cyanide conversion using photocatalysis and ozonation**

Photocatalysis is an oxidation process that uses light especially UV-irradiation and oxides such as hydrogen peroxide ($H_2O_2$) and titanium dioxide ($TiO_2$) to degrade cyanide. The oxides dissociate in the waste solution to produce a hydroxyl radical ($OH^-$) which degrade the cyanide to carbon dioxide and carboxylic acids with intermediate cyanate and ammonium-nitrogen (Equations 2.12 & 2.13).

$$CN^- + H_2O_2 \rightarrow CNO^- + H_2O \qquad\qquad (Eq.\,2.12)$$

$$CNO^- + 2H_2O \xrightarrow{H_2O_3} NH_4 + CO_3^{-2} \qquad\qquad (Eq.\,2.13)$$

Oxidation of CN by $H_2O_2$ is a slow and an unstable process, with high $H_2O_2$ dose requirements in irradiated wastewater and copper may be needed to increase the rate of degradation leading to increased operational costs hence, the general preference for titanium oxide (Sarla *et al.*, 2004). However, $TiO_2$ produce a protonated species, $HO_2^-$, which cannot form $OH^-$ (Equation 2.14). In order to cater for this, ozone ($O_3$) is required (Equation 2.15) and within a single step, a hydroxyl radical ($OH^-$) can be produced (Klare *et al.*, 2000).

$$O_2 + e^- \rightarrow O_2^- \xrightarrow{H^+} HO_2^- \qquad\qquad (Eq.\,2.14)$$

$$O_3 + e^- \rightarrow O_3^- \xrightarrow{H^+} HO_3^- \rightarrow O_2 + OH^- \qquad\qquad (Eq.\,2.15)$$
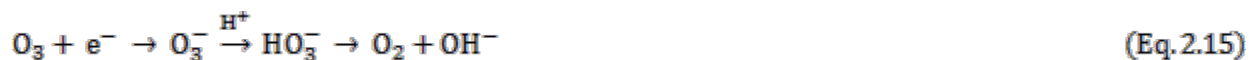
The advantages of ozone enhanced photocatalysis are: (a) no residual toxic chemicals are formed; (b) equipment low maintenance requirements; (c) the operation is simple and (d) rapid and complete degradation of free cyanides and its complexes through several reaction routes (Carrillo-Pedroza *et al.*, 2000; Hernández-Alonso *et al.*, 2002). As with other oxidation processes, oxidation by-products are produced.

Cape Peninsula
University of Technology

### 2.4.3 Biological treatment of cyanides in wastewater containing heavy metals

In response to increasing environmental challenges of removing hazardous waste, attention has shifted to the use of biomaterials for the treatment of cyanides and heavy metals in wastewater. This is because biological processes are environmentally friendly, cost effective and the remediation can be done *in-situ* at the wastewater treatment plant.

### 2.4.3.1. Cyanide biological treatment

Several species of fungi (*Aspergillus sp., Fusarium sp.*) and bacteria (*Bacillus sp., Pseudomonas sp.*) have been found to incorporate cyanide as a nutrient source for nitrogen and/or carbon via an ammonia ($NH_3$) intermediate (Mekuto *et al.*, 2013; Santos *et al.*, 2013a). Dubey and Holmes (1995) highlight the available pathway for enzymatic conversion of cyanide to ammonium-nitrogen using a variety of microorganisms.

- Cyanide hydratase –cyanide is degraded using cyanide hydratase leading to the formation of methanamide and thus converted into carbondioxide ($CO_2$) and $NH_3$, the reaction is irreversible. An example of such an organism involved in this process is *Pseudomonas sp.*

- Cyanoalanine synthase – oxygen ($O_2$) requirement is indirect and $CO_2$ is not released. Cyanide is converted into β-cyanoalanine or α-aminonitrile, catalyzed by β-cyanoalanine synthase. The product is hydrolysed to release ammonium-nitrogen and an acid. An example of such an organism involved in this process is *Bacillus megaterium.*

- Cyanase – the enzyme cyanide monoxygenase is used to convert HCN to cyanate (HOCN) which decomposes through cyanase to produce $CO_2$ and ammonium-nitrogen. An example of such an organism involved in this process is *Escherichia coli.*

- Cyanidase – cyanide is converted into formate and ammonium-nitrogen through a cyanidase catalyst. This enzyme's resistance to metallic-cyanide complexes, alcohols and nitriles makes it a preferred pathway. It is very active in the presence or absence of oxygen and coenzyme generation is not required. An example of such an organism involved in this process is *Alcaligenes sp.*

- Rhodanese – the use of rhodanese which activity is regulated by divalent ions and phosphate ions convert cyanide into less toxic cyanide species. It functions optimally at a pH range of 8.5 to 11.5 and at temperature between 35°C to 55°C. The *Bacillus stearothermophilus* can degrade cyanide in to a thiocyanate form.

Cyanidase and rhodanase are recommended for treatment of wastewater contaminated with metallic cyanides; however, only cyanidase has been used under experimental field conditions (Dubey *et al.*, 1995). The shortcoming of the cyanide enzymatic conversion system is that it may be affected by factors such as, initial cyanide concentration, pH, temperature, tolerance of the organisms to cyanide, and the presence of heavy metal ions in the wastewater (Gupta *et al.*, 2010). Cyanide is a respiratory inhibitor; i.e. it binds with a terminal electron acceptor of the enzymes; thus it reduces the respiration rate of the microorganism (Wagner *et al.*, 1995; Barclay *et al.*, 1998). In response to ATP synthesis inhibition, an alternative pathway is used for ATP production hence increasing the energy requirements for cellular respiration by the microorganism (Siedow *et al.*, 1995). The entire pathway depends on the mechanism used by the microorganism to tolerate the cyanide. For *Fusarium oxysporum*, its multienzyme production abilities and capability to use a broad range of cyano-compounds as substrates would help navigate this challenge (Cluness *et al.*, 1993; Anuradha *et al.*, 2010).

## 2.4.3.2 Effect of heavy metals in biological treatment of cyanide

The application of microorganism for heavy metal laden wastewater treatment requires the uptake of these metals; however, the sorption capacities and affinities of living and non-living microorganism differs. The metals are accumulated (bioaccumulation) on the cell wall of the organism during metabolic processes. The binding of the metals to the biomaterial (biosorption) depends on the functional groups on the cell wall. Wang and Chen (2009) gave the summary of different functional groups and their respective metal affinity in Table 2.4.

**Table 2. 4: Ligands in biomaterials and three classes of metals (Wang *et al.*, 2009)**

| Ligand type | Ligands | Metals |
|---|---|---|
| I: Type A | $SO_4^-$, $ROSO_3^-$, $F^-$, $O^{2-}$, ROR $OH^-$, $H_2O$, $CO_3^{2-}$, $NO_3^-$, $HPO_4^{2-}$, $PO_4^{3-}$, ROH, $RCOO^-$, C=O, | Ca, Sc, Rb, Sr, Y, Cs, Li, Be, Na, Mg, K Ba, La, Fr, Ra, Ac, Al, Actinides, Lanthanides. |
| II: Type B | $H^-$, $R_3As$ $I^-$, $R^-$, $S^{2-}$, $RS^-$, $R_2S$, $CN^-$, CO, | Rh, Pb, Bi, Pd, Ag, Lr, Hg, Tl, Pt, Au. |
| III: Type C/Borderline ions | $N_3^-$, $NO_2^-$, $RNH_2$, -CO-N-R, $O_2$, $R_2NH$, $R_3N$, =N-, $O_2^-$, $NH_3$, $N_2$, $O_2^{2-}$, $Cl^-$, $SO_3^{2-}$, $Br^-$. | Sn, Sb, As, Mn, Fe, Co, Ni, ,Ti, V, Cr, Cu, Zn, Ga, Cd, In. |

The symbol *R* represents an alkyl radical such as $CH_2$-, $CH_3CH_2$- etc. Type A metal ions readily bind the ligands of Type I. Type B ions show preference for Type II ligands but also form strong bonds with Type C (III) ligands. Type C metal ions could bind to any of the three classes of ligands depending on the associate affinity. The functional groups of the living biomass differ due to the composition of the nutrient media used leading to a change in sorption capability as the biomass ages (Eccles, 1995; Wang *et al.*, 2009).

Non-living microbial biomass often exhibit a higher affinity for metal ions than living cells due to the absence of competing protons produced in living cells (Illán-Cabeza *et al.*, 2008; Acheampong *et al.*, 2010). The non-living biomass is preferred to living biomass for metal uptake because; (a) it requires no growth media and nutrients; (b) a large quantity of biomass can be easily obtained; (c) the process is uncomplicated compared with ion-exchange; (d) it is independent of toxicity limitations; (e) there is a near infinite supply and shelf life for biomaterials; (f) unused or surplus biomass can be used for biosorption.

Factors other than living microorganisms affecting the metal sorption efficiency are of great interest. This is influenced by such factors like initial metal concentration, sorbent dosage and size, temperature and pH including the presence of inhibitory pollutants such as cyanide (Acheampong *et al.*, 2010). The solution pH is the most crucial factor that influences the redox reaction, ligand class complexation, hydrolysis, metal speciation, etc. At low pH (pH ≤ 4), the metal removal efficiency by the biomaterial is observed to be low due to the competition between metal (cations) and hydrogen ions (protons). For example, Dakiky *et al.* (2002) removed Cr(VI) using sawdust at optimum pH of 2 with 15.8% efficiency and Babel *et al.* (2004) achieved removal efficiency of 54% for Cr(VI) at pH of 4 using coconut shell carbon treated with nitric acid. As the pH increases, the removal efficiency increases due to increased ion exchange within the solution. At pH near neutral ( 6 ≤ pH ≤ 8), Gherbi *et al.* (2004) removed Cu(II) with an efficiency 84% using a calcinated wheat by-product at optimum pH 6; however, Yu *et al.* (2000) removed Cu(II) at pH 8 on sawdust achieving only 19% efficiency. This reduction was due to the competition between the cations and protons at equilibrium. The ion-exchange then increases rapidly as the pH increases (pH ≥ 8) as a result of less competition between the cations and protons leading to improved efficiency (Eccles, 1995; Igwe *et al.*, 2007). This indicates that pH

Cape Peninsula
University of Technology

plays a crucial role in the sorption of metal pollutants, which means that, when cyanide degradation studies are conducted in the presence of heavy metals, pH must be monitored.

Another factor of great importance is temperature, which affects cellular wall functions. Temperature change alters cellular metabolic functions and the biomass-environment interaction. Therefore, it was imperative to monitor the effect of temperature during cyanide degradation (Acheampong *et al.*, 2010; Murthy *et al.*, 2012).

## 2.5 Application of *Fusarium* species and agricultural waste

### 2.5.1 *Fusarium oxysporum* in cyanide biodegradation

A number of different studies have been carried out to study fungal species which degrades cyanide. White rot fungi, *Trametes versicolor* have been shown to tolerate cyanide up to 130 mg F-CN/L with complete degradation within 42 hours to produce small quantities of ammonium-nitrogen (5.24 mg $NH_4^+$-N/L) (Cabuk *et al.*, 2006). Similarly, fourteen (14) fungi capable of degrading cyanide, were examined by Pereira *et al.* (1996), of which only *Fusarium oxysporum* was found to tolerate cyanide concentration up to 520 mg F-CN/L; however, cyanide volatilised was not mentioned nor quantified in this study. Furthermore, studies of microbial species capable of degrading cyanide are shown in Table 2.5.

*Fusarium* species are widely distributed in different soils and plants. They can be incubated at 25°C under light and 20°C in darkness alternatively for optimum growth ((Leslie *et al.*, 2006)). They cause spoilage of agricultural products and produce mycotoxins in cereal crops that affect human and animal health if the toxins enter the food-chain. Despite this, *Fusarium sp.* has been found useful in the hydrolysis of starch. The hydrolysis of agricultural produce is due to the production of extracellular ezymes such as pectinase, cellulase, xylanase, amylase, and organic acids (Mitchell *et al.*, 2006; Dogaris *et al.*, 2009). *Fusarium sp.* is also known for the production of cyanide hydratase, nitrilase and cyanidase enzymes in the presence of cyanide. These enzymes have been identified to degrade cyanides through hydrolysis by cyanidase and nitrilase to ammonium-nitrogen and carboxylic acid at varying temperature and pH, with the fungus metabolising the by-products as nitrogen and/or carbon sources, respectively (O'Reilly *et*

*al.*, 2003; Gupta *et al.*, 2010). The cyanide hydratase converts hydrogen cyanide to amide by-products (e.g. methamide) and ammonium-nitrogen while two molecules of water react with nitrilase to hydrolyse R-CN to produce a carboxylic acid (Nolan *et al.*, 2003). *Fusarium sp.* also produces nitrilase hence the need to focus on nitrilase activity. The nitrilase from *Fusarium oxysporum* can be a controlling factor in the reaction mechanism used for cyanide conversion (Kobayashi *et al.*, 2000). Compared with other enzymes derived from bacteria, nitrilase and cyanidase enzymes are of higher activity and can degrade various cyanides (Rao *et al.*, 2010).

The degradation of cyanide by these enzymes is aided by glutamate, cysteine, and lysine. The nucleophilic attack on the cyano-carbon by the cysteine group causes a covalent enzyme-thioimidiate complex formation which is then hydrolysed to give a tetrahedral intermediate. This intermediate, rapidly breaks-down via the separation of the carbon-nitrogen bond producing ammonium-nitrogen. Another water molecule can be deprotonated, which can launch a nucleophilic attack on the cyanide molecule to form a new tetrahedral intermediate. Carboxylic acids can then be produced through the decomposition of the tetrahedral intermediate (see Figure 2.5). *Fusarium sp.* was identified as having a new pathway for respiration which assists in tolerance of higher cyanide concentrations that would inhibit respiration in other microorganisms (Martínková *et al.*, 2009). The mechanism for cyanide conversion using nitrilase and/or cyanidases is shown in Figure 2.5.

Cape Peninsula
University of Technology

**Table 2. 5: Cyanide degrading microbial species**

| Microorganism | C-source | N-source | Temperature (°C) | pH | Reference |
|---|---|---|---|---|---|
| *Agrobacterium tumefaciens* | Starch | KCN | - | 7.2 | (Potivichayanon *et al.*, 2014) |
| *Aspergillus awamori* | *Citrus sinesis* extract | KCN | 40 | 8.84 | (Santos *et al.*, 2013a) |
| *Baccillus pumilus* | Glucose | KCN | 40 | 8.5-9 | (Skowronski *et al.*, 1969) |
| *Baccillus stearothermphilus* | - | NaCN | 27±2 | 7.8 | (Atkinson, 1975) |
| *Burkholderia cepacia* | Fructose, glucose, mannose | KCN | 30 | 10 | (Adjei *et al.*, 2000) |
| *Citrobacter sp., Pseudomonas sp.* | Sugarcane molasses, glucose | $[Cu(CN)_4]^{2-}$, $[Zn(CN)_4]^{2-}$ | 35 | 7.5 | (Patil *et al.*, 2000) |
| *Cryptococcus humicolus* MCN2 | Glucose | KCN | 25 | 7.5 | (Kwon *et al.*, 2002) |
| *Eschericia coli* | Glucose | KCN | 30 | 9.2 | (Figueira *et al.*, 1996) |
| *Fusarium solani* | Glucose | $K_2Ni(CN)_4$, KCN | 25 | 7.0 | (Barclay *et al.*, 1998) |
| *Fusarium solani* | Yeast | KCN | 30 | 9.2-10.7 | (Dumestre *et al.*, 1997) |
| *Fusarium oxysporum* | Glucose | KCN | 25 | 8.0 | (Pereira *et al.*, 1996) |
| *Fusarium oxysporum* immobilised on sodium alginate | Formamide | Cyanides | 25-30 | 8 | (Campos *et al.*, 2006) |

**Cont.: Table 2.5**

| | | | | | |
|---|---|---|---|---|---|
| *Gloeocercospora sorghi*, *Stemphylium loti* | Glucose | KCN | 35, 28 | 5.3-5.7, 7.0 | (Nazly *et al.*, 1983) |
| *Klebsiella oxytoca* | Glucose | KCN | 30 | 7 | (Kao *et al.*, 2003) |
| *Klebsiella oxytoca immobilised cell* | Alginate and cellulose triacetate | KCN | 30 | 7 | (Chen *et al.*, 2008) |
| Mixed culture of bacteria | Glucose | $CN^-_{WAD}$ | 22 | 7.0 | (White *et al.*, 1998) |
| Mixed culture of bacteria immobilised on ultrafiltration membranes | Phenol | Cyanides | 25 | - | (Kowalska *et al.*, 1998) |
| *Pseudomonas fluorescens* | Glucose | Ferrocyanide | 25 | 5 | (Dursun *et al.*, 1999) |
| *Pseudomonas fluorescens* immobilised on calcium alginate | Glucose | Ferrocyanide | 25-35 | 4-7 | (Dursun *et al.*, 2000) |
| *Pseudomonas fluorescens* immobilised on zeolite | Zeolite | Tetra-cyano-nickelate (II) | 30 | - | (Suh *et al.*, 1994) |
| *Pseudomonas pseudoalcaligenes CECT5344* | $CH_3COONa$ | NaCN | 30 | 9.5 | (Luque-Almagro *et al.*, 2005) |
| *Pseudomonas putida* BCN3 | Glucose | $[K_2[Ni(CN)_4]]$ | 30 | - | (Silva-Avalos *et al.*, 1990) |
| *Pseudomonas putida* immobilised on sodium alginate | NaCN | NaCN | 25 | 6.7 | (Babu *et al.*, 1992) |
| *Pseudomonas putida* immobilised on sodium alginate | NaCN, sodium alginate | NaCN, Cyanates and thiocyanates | 25 | 7.5 | (Chapatwala *et al.*, 1998) |

**Cont.: Table 2.5**

| | | | | | |
|---|---|---|---|---|---|
| *Pseudomonas stutzeri* AK61 | - | KCN | 30 | 7.6 | (Watanabe *et al.*, 1998) |
| *Pseudomonas sp.*(CM5, CMN2) | Glycerol | $CN^-_{WAD}$ | 30 | 9.2-11.4 | (Akcil *et al.*, 2003) |
| *Stemphilium loti* | | KCN | 25 | 6.5, 7.5 | (Fry *et al.*, 1972) |
| *Trametes versicolor* | Citrate | KCN | 30 | 10.5 | (Cabuk *et al.*, 2006) |
| *Trichoderma sp.* | Glucose | $CN^-$ | 25 | 6.5 | (Ezzi *et al.*, 2005) |
| *Scenedesmus obliquus* | NaCN | NaCN | - | 10.3 | (Gurbuz *et al.*, 2009) |
| *Rhodococcus* UKMP-5M | Glucose | KCN | 30 | 6.6 | (Maniyam *et al.*, 2011) |

30

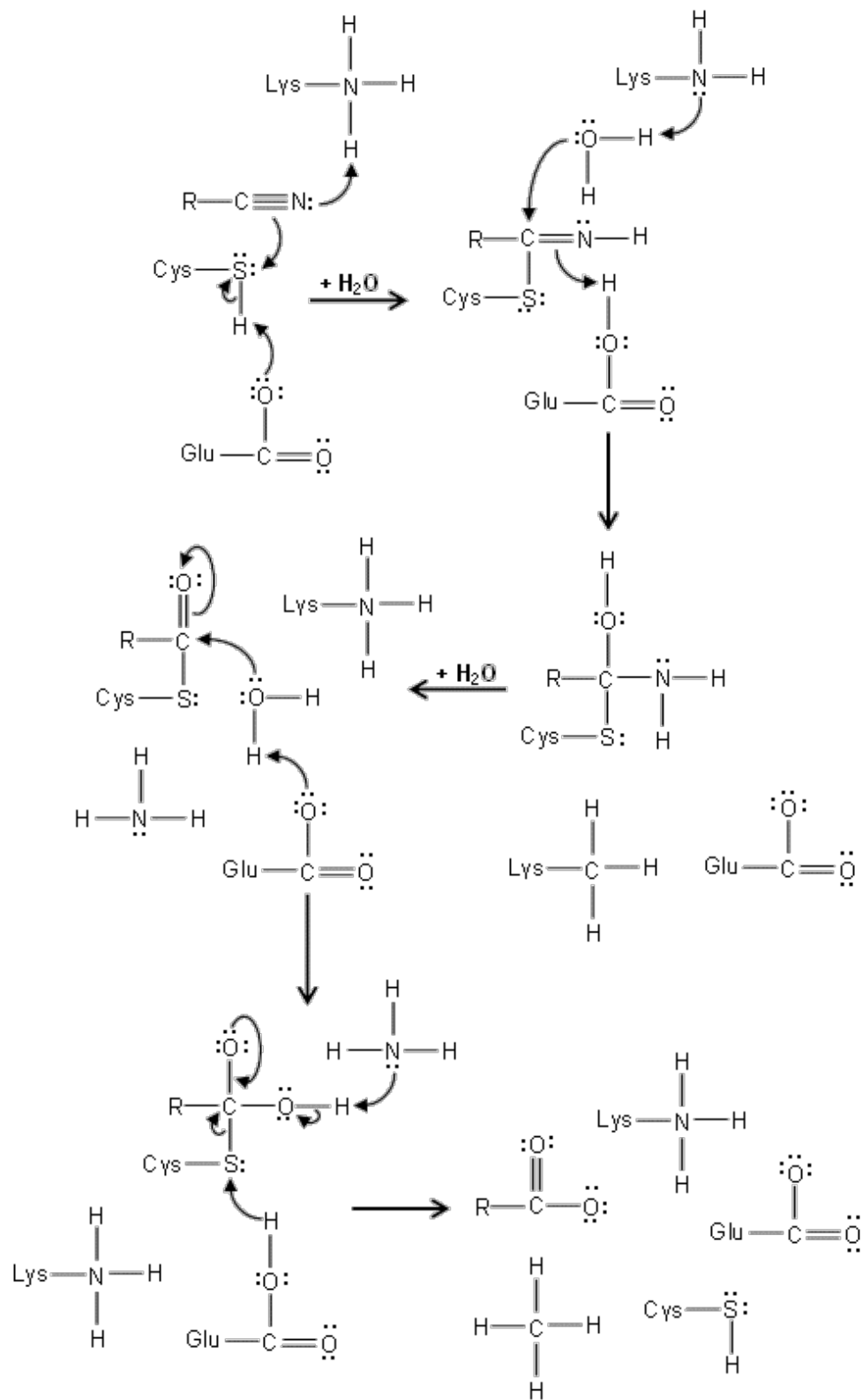Cape Peninsula
University of Technology

**Figure 2. 5: Enzymatic conversion of cyanide using nitrilase or cyanidase (Santos, 2013)**

## 2.5.2 Bioprocess application of agricultural waste in cyanide degradation

Agricultural waste consist of water, reducible sugars, minerals and proteins which makes it suitable for the growth of microorganisms and as feedstock for the production of value added products such as biofuel, enzymes, biosurfactants, amino acids, aromatic compounds, etc.; however, limited studies have shown its potential as a feedstock and solid support in a bioreactor for the biodegradation of cyanide in the presence of heavy metals (Mussatto *et al.*, 2012). Hydroxyl functional groups found in agricultural wastes can act as pseudo-catalysts for the conversion of free cyanide to ammonium-nitrogen. Though, the free hydroxyl functional group is a weak acid, they are able to deprotonate to produce alkoxides in the presence of a strong base such as cyanides especially at high alkaline pH (see figure 2.6). Santos *et al.* (2013b) used *Citrus sinesis* instead of refined carbon source for *Aspergillus awamori* growth in a bioprocess used for the degradation of cyanide. The authors discovered that the hydrolysed citrus peel was more efficient (63%) than unhydrolysed citrus peel (18%). However, cyanide degradation was inhibited by the presence of metals in the wastewater due to competition between cyanide and metals for the hydroxyl group present within the citrus peel. The hydroxyl groups present within *Beta vulgaris* (Wootton-Beard *et al.*, 2011; Ntwampe *et al.*, 2013) could be an added advantage when used with *Fusarium oxysporum* for the bioremediation of cyanide in gold mine wastewater, a concept that needs further investigation. Other applications of agricultural waste are shown in Table 2.6.

**Table 2. 6: Agricultural waste application in bioprocess (Mussatto *et al.*, 2012; Ntwampe *et al.*, 2013)**

| Microorganism | Solid Support |
|---|---|
| *Gibberella fujikuroi, Fusarium moniliforme* | Corn cob, sugar cane bagasse, cassava flour |
| *Bacillus thuringiensis* | Coconut waste |
| *Penicillium chrysogenum* | Sugarcane bagasse |
| *Streptomyces rimosus* | Corn cob |
| *Streptomyces viridifaciens* | Sweet potato waste |
| *Aspergillus awamori* | Orange peel, carrot, onion, and apple |
| *Streptomyces sp* | Coffee pulp waste |
| *Monascus purfureus* | Sugarcane bagasse |

| | |
|---|---|
| *Bacillus safensis, lichenformis and tequilensis* | Whey, Rice straw |
| *Aspergillus niger* | Wheat bran, soybean cake waste |
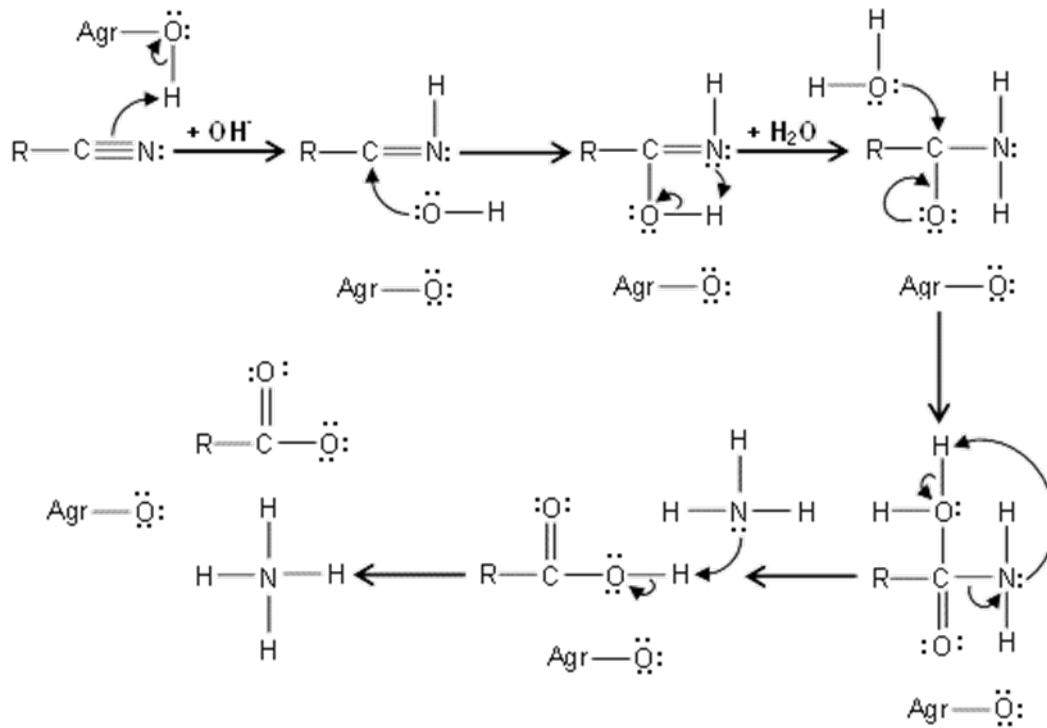


**Figure 2. 6: Pseudo-catalyst conversion of cyanide by free hydroxyl functional group (Santos, 2013)**

# CHAPTER 3

# MATERIALS AND METHODS

# CHAPTER 3
# MATERIALS AND METHODS

## 3.1 Introduction

This chapter lists the materials, experimental procedures and the rationale for each procedure used for this study. Furthermore, analytical tools used are also discussed. All experiments and analysis were duplicated in batch cultures using airtight, multiport, round-bottom flasks with a sampling port.

## 3.2 Gold mine wastewater sample

Wastewaters from gold mine process effluent and tailings dam have been shown to contain high metal concentrations. Acheampong *et al.* (2013) in his analysis of a gold mine effluent in Ghana showed high cyanide concentration and cyanide-metal complexes in both process effluent and tailing dams. The wastewater sample used in this study was adapted from his analysis as shown in Table 3.1 with varying cyanide concentrations of 100, 200, 300 and 500 mg F-CN/L including metals, arsenic (7.1 mg/L), iron (4.5 mg/L), copper (8 mg/L), lead (0.2 mg/L) and zinc (0.2 mg/L).

**Table 3. 1: Wastewater composition (Acheampong *et al.*, 2013)**

| Parameters | Values |
|---|---|
| Temperature | 33.5 (± 1.5)°C |
| pH | 10.3 (± 1.0) |
| Arsenic (As) | 7.1 (± 2.6) mg/L |
| Iron (Fe) | 4.5 (± 6.6) mg/L |
| Copper (Cu) | 8.0 (± 3.9) mg/L |
| Lead (Pb) | 0.2 (± 0.3) mg/L |
| Zinc (Zn) | 0.2 (± 0.5) mg/L |
| Ammonium ( $NH_4^+$) | 53.4 (± 22.7) mg/L |
| Phosphate ($PO_4^{3-}$) | 13.3 (± 5.6) mg/L |
| Cyanide | 66.7 (±33.5) mg/L |

Cape Peninsula
University of Technology

## 3.3 Inoculum Preparation

Isolated *Fusarium oxysporum* from cyanide containing pesticides was re-cultured on potato dextrose agar (PDA) plates, parafilmed and incubated at 25°C for 5 days. Sterile distilled water was added to each plate and spatula was used to harvest the spores and mycelium from the plate. In order to separate the spores from mycelia, the spore-mycelium suspension was filtered through a glass wool using 20 mL syringes to entrap the mycelium unto the glasswool and filtrate spore was collected and stored at 4°C. The procedure was repeated for each plate. In addition, spore concentration was quantified by a series of dilution using sterile distilled water and the spore solution. A Jenway 6715 UV/Visible spectrophotometer was used at varying wavelength to determine the maxima absorbance for the spore dilution. The absorbance was found at a maximum of 300 nm. The spore concentration and absorbance of each of the spore dilutions was determined in duplicate using both a direct counting system in a Marienfeld Neubauer cell-counter and a Nikon Eclipse E2000, phase contrast 1 and 100 × magnification. The calibration curve for the spore concentration was determined by plotting absorbance versus the spore concentration (see Figure 3.1).
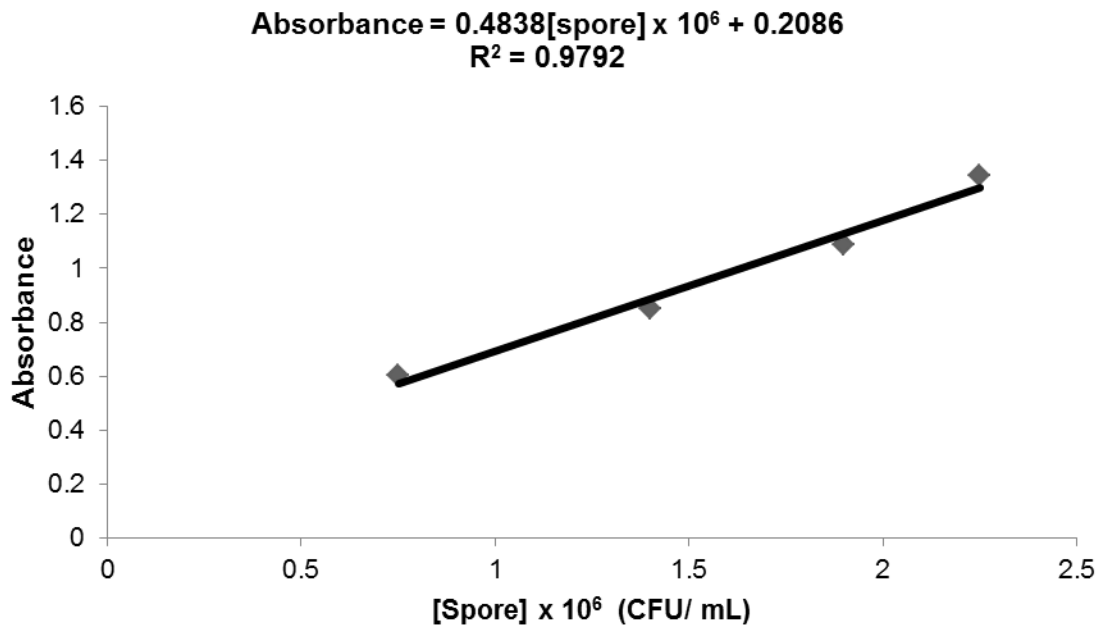


**Absorbance = 0.4838[spore] x $10^6$ + 0.2086**
**$R^2$ = 0.9792**

**Figure 3. 1**: **Calibration curve for *Fusarium oxysporum* spore concentration**

## 3.4 Agro-waste preparation

*Beta vulgaris* (beetroot) obtained from fruit and vegetables company, Cape Town, South Africa was used in the experiments. It was dried at 80°C for seven days and milled to less than 100 µm using a grinder (Bosch MKM 7000). The agro-waste was mixed with distilled water and autoclaved at 116°C for 15 minutes and then cooled to room temperature. The solution was filtered through a No. 1 Whatman filter paper in a Brüchner funnel under vacuum and the filtrate was used for the experiments.

## 3.5 Experimental design

The strategy of the first set of experiments was based on a response surface method (RSM). Response surface methodology is a statistical tool for design of experiment to determine optimum responses within a range of factors leading to peak process performance at minimal cost ((Anderson *et al.*, 2005)). A central composite design (CCD) was used in this study to assess two variables; temperature and pH for the construction of a quadratic model response for the biodegradation of cyanide. The Design-Expert® software version 6.0.8 (Stat-Ease Inc., USA) was used to generate the experimental design. Each variable was analysed at five different coded levels; -β, -1, 0, +1, and +β, representing a factorial, centre and axial points (Table 3.2). A fourteen (14) run experimental plan was developed, consisting of four factorial points ($2^k$, where k is the number of variables) six centre points (level 0) and four axial points representing the outlier points on the axis of each variable at distance +β from high level (+1) and –β from low level (-1) (where $\beta = 2^{k/4} = 1.4142$). The experimental variables in coded values are shown in Table 3.3. Each sample was inoculated with 2% (v/v) of inoculum size in multiple-port airtight shake flasks to minimize volatilization of cyanide as a gas, i.e. cyanide stripping. The uninoculated broth served as a control under specified conditions. The pH of the samples was adjusted using 1 M NaOH or 1 M HCl accordingly. All experiments were in duplicate and the mean of the measured values was used to generate the response (*Y*), which was the cyanide biodegradation. The response (*Y*) of the biodegradation process was represented by the quadratic model:

$$Y = \alpha_o + \sum_{i=1}^{n} \alpha_i X_i + \sum_{i=1}^{n} \alpha_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \alpha_{ij} X_i X_j + \varepsilon \qquad (Eq.3.1)$$

Cape Peninsula
University of Technology

Where $X_i, X_j \ldots\ldots, X_n$ were independent variables, $\varepsilon$ was the randomised error, $\alpha_o$ was the offset term, $\alpha_i, \alpha_{ii}$ $and$ $\alpha_{ij}$ were linear, squared, and interaction effects, respectively.

**Table 3. 2: Media components in CCD experiments and corresponding concentration levels**

| Variables | Units | Code | High levels (+1) | Medium levels (0) | Low levels (-1) |
|---|---|---|---|---|---|
| Temperature | °C | A | 30 | 19.5 | 9 |
| pH | - | B | 11 | 8.5 | 6 |

**Table 3. 3: Coded experimental design variables**

| Run | A | B |
|---|---|---|
| 1 | 0 | 0 |
| 2 | -1 | 1 |
| 3 | 0 | 0 |
| 4 | 1 | 1 |
| 5 | 1 | -1 |
| 6 | 0 | 0 |
| 7 | -1 | -1 |
| 8 | 0 | 0 |
| 9 | 0 | β |
| 10 | β | 0 |
| 11 | -β | 0 |
| 12 | 0 | -β |
| 13 | 0 | 0 |
| 14 | 0 | 0 |

A and B represent the coded level of variables while β represents the axial point with coded level of 1.4142

Cape Peninsula
University of Technology

## 3.6 Experimental procedures

### 3.6.1 Free cyanide biodegradation by *Fusarium oxysporum* grown on *Beta vulgaris*

Synthetic goldmine wastewater of 20 mL with 1 mL spore solution (2.25 x 10$^6$) of *Fusarium oxysporum* was added to 10 mL treated *Beta vulgaris* of concentration 0.0098 g/mL as the sole carbon and energy source followed by incubated for 48 h in a rotary shaker at 70 rpm to release the carbohydrates into the solution at desired operating conditions as specified in Table 3.3. The synthetic wastewater had similar features with that of Acheampong *et al.* (2013) particularly with regards to metal content. Afterwards, 20 mL of dissolved KCN in distilled water was added to the flask to make a culture with a final cyanide concentration of 500 mg CN$^-$/L and culture volume of 51 mL. Subsequently, the culture broth was incubated for further 72 h in a temperature controlled rotary shaker (ZHCHENG model ZHYWY-1102) at 70 rpm. Free cyanide volatilization was accounted for using Equation 3.2 and 3.3.

$$CN_B^- = CN_I^- - CN_R^- - CN_V^- \qquad (Eq. 3.2)$$

$$CN_V^- = CN_{IC}^- - CN_{FC}^- \qquad (Eq. 3.3)$$

Where $CN_B^-$ was the free cyanide bioremediated; $CN_I^-$ was the initial free cyanide concentration in the culture broth; $CN_R^-$ is the residual free cyanide concentration measured after incubation; $CN_V^-$ was the free cyanide that volatilized during culture incubation; $CN_{IC}^-$ was the initial free cyanide concentration in control culture (500 mg CN$^-$/L); $CN_{FC}^-$ was the final free cyanide concentration in the control culture. The control was prepared under the same conditions as other cultures except for the absence of *Fusarium oxysporum*.

### 3.6.2 Biodegradation kinetics study using *Fusarium oxysporum*

Subsequent to the determination of optimal operating condition for the biodegradation of cyanide in the presence of heavy metals (see sections 3.5 and 3.6.1_, the growth kinetics of *Fusarium oxysporum* and free cyanide degradation kinetics were studied under these conditions. 20 mL volume of the *Beta vulgaris* waste extract (13% v/v) was used in the culture with 1 mL cell concentration of 0.7 % (v/v). A volume of 49 mL of synthetic wastewater with 80 mL of dissolved potassium cyanide in phosphate buffer was added to the flask to make a final

Cape Peninsula
University of Technology

volume of 150 mL. The cultures were incubated at predetermined optimal temperature and pH (determined in section 3.6.1) in a shaking ZHCHENG incubator (model ZHYWY-1102) at 70 rpm for a week. Similarly, F-CN volatilization was quantified using Equations 3.2 and 3.3. The cultures contained 100, 200 and 300 mg $CN^-$/L. Another culture was prepared without cyanide to monitor the growth of *Fusarium oxysporum,* under similar conditions as those used for cyanide containing cultures.

## 3.7 Analytical methods

### 3.7.1 Sample preparation

Samples were collected after 72 h in section 3.6.1 and every 24 h in section 3.6.2. All samples were centrifuged at 13000 rpm for 5 minutes using a Haraeus Megafuge 1.0 before analysis commenced.

### 3.7.2 Photometric tests

Cyanide ($CN^-$) (09701), ammonium ($NH_4^+$) (00683) and nitrate (14773) kits supplied from MERCK® were used to quantify the concentration of free cyanide, ammonium and nitrate using a NOVA 60 Spectroquant at absorbance of 0.010 A. The cyanide test kit measures cyanide as free cyanide via the reaction of a chlorinating agent (chloramine-T), 1, 3-dimethylbarbituric acid and cyanide, to form a violet dye that is quntified photometrically. The maximum error of measurement was ± 0.013 mg F-CN/L. The ammonium test kit function is based on the reaction of ammonia with chlorinating agent (hypochlorite ions) and thymol to form indophenol that was photometrically determined. The ammonium was measured as ammonium-nitrogen with a maximum accuracy of ± 0.08 mg $NH_4$-N/L. The nitrate test kit measures nitrate as nitrate-nitrogen in the reaction of concentrated sulphuric acid with a benzoic acid derivative. A red nitro compound which can be determined photometrically is formed (accuracy of ± 0.6 mg $NO_3$-N/L).

Cape Peninsula
University of Technology

### 3.7.3 Quantitation of *Fusarium oxysporum* spore concentration

*Fusarium oxysporum* growth was quantified by a series of dilutions using sterile distilled water and centrifuged sample. Absorbance was measured on a Jenway 6715 UV/Visible spectrophotometer. The corresponding cell concentration was estimated using Figure 3.1.

### 3.7.4 Modelling *Fusarium oxysporum* growth

The growth kinetic was analysed by estimating the specific growth rate, $\mu$ ($h^{-1}$), and doubling time $t_d$ (h) using Equations 3.4 and 3.5 with an assumption of first order kinetics (Doran, 1995).

$$\ln X_2 = \ln X_1 + \mu(t_2 - t_1) \qquad (Eq.\,3.4)$$

$$t_d = \frac{\ln 2}{\mu} \qquad (Eq.\,3.5)$$

Where $X_1$ and $X_2$ are the cell concentrations at time $t_1$ and $t_2$ respectively, $t_d$ is the doubling time in h when $X_2 = 2X_1$

The mechanism of the cell growth is simplified by Monod's kinetic model in Equation 3.6.

$$\mu = \frac{\mu_m S}{K_S + S} \qquad (Eq.\,3.6)$$

Where $\mu_m$ ($h^{-1}$) is the maximum specific growth rate and $K_S$ (mg/L) is the saturation constant which are the Monod's model kinetic parameters. $S$ is the residual substrate concentration.

$$S = K_S \text{ When } \mu = \frac{\mu_m}{2} \qquad (Eq.\,3.7)$$

### 3.7.5 Free Cyanide biodegradation kinetics

The order of a reaction can be determined when the mechanism of the reaction is known. The mechanism highlights the steps from reactants leading to the formation of products. The rate of each step differs and the overall reaction will be decided by the slowest step. Reaction rate is proportional to the concentration of reactants raised to an appropriate power (Upadhyay, 2006). Assuming a reaction pP + qQ → rR + sS, the rate in term of the disappearance of P is:

$$r_p = -\frac{dC_p}{dt} = K\, C_p^{\alpha}\, C_Q^{\beta} \tag{Eq. 3.8}$$

Where $\alpha$ is the order of reaction with respect to P and $\beta$ is the order of reaction with respect to Q. $K$ is the reaction rate constant which is independent of the cyanide concentration but depends largely on temperature.

For this study, a first order kinetics was assumed, i.e.:

$$\frac{d[CN]}{dt} = -k[CN] \tag{Eq. 3.9}$$

Which gives (Eq. 3.9):

$$\ln\frac{[CN]_2}{[CN]_1} = -k\,(t_2 - t_1) \tag{Eq. 3.10}$$

Where $[CN]_2$ and $[CN]_1$ are the concentrations (mg/L) of free cyanide at time (h) $t_2$ and $t_1$ respectively. $k$ is the rate of biodegradation (h$^{-1}$). Equation 3.10 was used in an Ordinary Differential Equation (ODE) solver, to simulate the cyanide degradation kinetics. Since the reaction rate constant is temperature dependent, a new set of experiments similar to those described in section 3.6.2. were performed at 5°C to represent the temperature drop such as in South Africa in winter for the examination of the effect of cold temperature conditions on free cyanide biodegradation kinetics. The optimal operating condition determined in section 3.6.1 represented mostly the warm (summer) conditions.

Furthermore, a second model based on the Arrhenius law can be developed to show the effect of temperature on a biochemical reaction. As the temperatures increases, the cyanide solution molecule velocity also increases. This leads to an increase in the kinetic energy of the molecules. The minimum energy needed for the bioreaction to occur, i.e. activation energy, $E$, was calculated for the cyanide cultures using Arrhenius equation;

$$k = A\, e^{-E/RT} \tag{Eq. 3.11}$$

Cape Peninsula
University of Technology

Where $A$ is the frequency factor, $R$ is the gas constant ($R$ = 8.314 J/K mol), $T$ is the temperature in Kelvin and $E$ is the activation energy of the biodegradation reaction (kJ/mol). Taking the natural logarithm of both sides of Equation 3.11, we have:

$$\ln k = \ln A - \frac{E}{RT} \qquad (Eq.\,3.12)$$

A plot of $\ln k$ against $1/T$ gives a straight line graph with a gradient of $-E/R$ and intercept on y-axis of $\ln A$. The activation energy was calculated using the optimum ($T_1$) and cold temperature conditions ($T_2$) with the rate constant being determined at each temperature. Denoting two different temperatures, $T_1$ and $T_2$, the rate constants, $k_1$ and $k_2$, Equation 3.12 becomes:

$$\ln k_1 = \ln A - \frac{E}{RT_1} \qquad (Eq.\,3.13)$$

and

$$\ln k_2 = \ln A - \frac{E}{RT_2} \qquad (Eq.\,3.14)$$

Subtracting equation 3.13 from 3.14 results in Equation 3.15:

$$\ln\left(\frac{k_1}{k_2}\right) = \left(\frac{1}{T_2} - \frac{1}{T_1}\right)\frac{E}{R} \qquad (Eq.\,3.15)$$

In this study, energy of activation for cyanide biodegradation was calculated using equation 3.14 with rate constant values determined at 5°C ($T_2$) and optimum temperature ($T_1$). Furthermore, the microorganism must be able to overcome the inhibitory effects of cyanide for any growth to occur. Thus, a higher energy is needed for cellular respiration for cyanide degradation. This energy is called activation energy for growth. As the temperature increases, the activity of the enzymes produced by the *Fusarium oxysporum* decreases due to enzyme denaturation and cell growth inhibition (Raybuck, 1992; Parolini *et al.*, 2010). Hence, energy of activation for *Fusarium oxysporum* growth during cyanide biodegradation was also estimated using the Arrhenius law as stated above.

Cape Peninsula
University of Technology

# CHAPTER 4

# RESULTS AND DISCUSSION

Cape Peninsula
University of Technology

## CHAPTER 4
## RESULTS AND DISCUSSION

### 4.1 Optimising biodegradation of cyanide using Response Surface Methodology

#### 4.1.1 Introduction

Response Surface Methodology (RSM) is a common tool for statistical design in biotechnology especially for determination of optimum process variables such as temperature, pH, and substrate concentration, among others. Two independent variables (temperature and pH) were examined in this study for their influence on cyanide biodegradation using central composite design (CCD), at a fixed *Beta vulgaris* extract concentration of 0.0098 g/mL.

#### 4.1.2 Aims

The aims were to:

- develop a model for cyanide biodegradation at cyanide concentration of 500 mg F-CN/L; and
- determine the optimum operating conditions for maximum cyanide biodegradation at cyanide concentration of 500 mg F-CN/L.

#### 4.1.3 Central Composite Design experimental response

A total of 14 experimental runs (in duplicate) were carried out as shown in Table 3.3. The results in Table 4.1 show a variation in the response of *Fusarium oxysporum* at 500 mg/L concentration of free cyanide after 72 hours. The highest F-CN degradation observed was 263 mg/L for run 9 which is an axial point (i.e. extremely high pH and moderate temperature). This could have been due to the microorganism using the cyanide as an energy source. Also, Santos *et al.* (2013) and Luque-Almagro *et al.* (2005) validated the high biodegradation of cyanide under a high pH with the application of *Aspergillus awamori* and *Pseudomonas pseudoalcaligenes* (CECT5344), respectively. The lowest F-CN biodegraded was 83 mg/L for run 11, also an axial point, i.e. at extremely low temperature an indication that the microorganism cannot effectively degrade cyanide at low temperature. Other researchers have shown that the growth of certain microorganisms is impeded at low temperature thereby resulting in the low removal of contaminants (Zilouei *et al.*, 2006; Zou *et al.*, 2014). There was considerable amount of F-CN

degradation in runs 1, 3, 4, 6, 8, 13 and 14 with varying concentration of residual ammonium-nitrogen and nitrates. These runs i.e., 1, 3, 4, 6, 7, 8, 13, and 14 experimental values had a 99% correlation with the predicted values indicating a high accuracy of the model used for predicting F-CN biodegradation.

**Table 4. 1: Coded experimental design variables and corresponding responses**

| Run | A | B | F-CN biodegradation (mg/L) | | Residual Ammonium-nitrogen (mg/L) | Residual Nitrate-nitrogen (mg/L) |
|---|---|---|---|---|---|---|
| | | | Experimental value | Predicted value | | |
| 1 | 0 | 0 | 239 | 238.86 | 210 | 38 |
| 2 | -1 | 1 | 229 | 196.59 | 100 | 108 |
| 3 | 0 | 0 | 239 | 238.86 | 210 | 38 |
| 4 | 1 | 1 | 250 | 250.29 | 40 | 98 |
| 5 | 1 | -1 | 135 | 167.62 | 320 | 140 |
| 6 | 0 | 0 | 239 | 238.86 | 210 | 38 |
| 7 | -1 | -1 | 127 | 126.92 | 128 | 176 |
| 8 | 0 | 0 | 239 | 239.14 | 210 | 38 |
| 9 | 0 | β | 263 | 285.75 | 210 | 38 |
| 10 | β | 0 | 196 | 172.77 | 100 | 39 |
| 11 | -β | 0 | 83 | 106.02 | 120 | 161 |
| 12 | 0 | -β | 201 | 178.03 | 30 | 36 |
| 13 | 0 | 0 | 239 | 239.14 | 210 | 38 |
| 14 | 0 | 0 | 239 | 239.14 | 210 | 38 |

### 4.1.4 Effect of pH on cyanide degradation by *Fusarium oxysporum*

The pH of the medium affected the biodegradation of free cyanide by the *Fusarium oxysporum* grown on *Beta vulgaris* waste –see Table 4.1. There was an increase in free cyanide biodegradation at pH 8.5 with the highest biodegradation occurring between pH 11 and 12.04,

for runs 4 (factorial point) and 9 (axial point), respectively. However, at axial points (Runs 10 and 11), the effect of an alkaline solution (pH 8.5) was inconsequential due to the extreme temperatures (4.65°C and 34.35°C). This study supported previous studies that showed that pH plays an important role in free cyanide degradation (Dash *et al.*, 2009a). At pH below 8, free cyanides exist in the form of HCN, which is highly stable in water thereby reducing the level of interaction between the free cyanide and the microorganism. In this study, it could be that the HCN at low pH impact on the interaction between *Beta vulgaris* and *Fusarium oxysporum* for multienzymes production as demonstrated by Anuradha *et al.* (2010). The initial cyanide concentration may have enhanced the influence of pH on the process. At initial cyanide concentration of 26 mg F-CN/L, (Huang *et al.*, 1977) showed that cyanide uptake was independent of pH at pH < 6, while there was direct pH dependency on cyanide degradation at pH between pH 7 and 11. Also, at a much lower initial cyanide concentration of 1.04 mg F-CN/L, the effect of pH was insignificant although an increment was observed between pH 8 and 9 (Guo *et al.*, 1993).

### 4.1.5 Effect of Temperature

The impact of temperature on free cyanide degradation using *Beta vulgaris* waste- *Fusarium oxysporum* cultures was observed to increase from 19°C to 30°C –see Table 4.1. Above 30°C, the biodegraded free cyanide was low (run 10). Below 10°C, free cyanide biodegradation was also generally low with a minimum being recorded at temperature of 4.65°C (Run 11). Previous researches have shown that cyanide degradation increases with an increase in temperature for most microorganisms except for a few such as *Pseudomonas sp.* (Adams *et al.*, 2001) where cyanide degradation reduced for increase in temperature from 5°C to 30°C. Generally, temperature affects cell constituents especially cell membrane and protein components, depending on the microbial growth rate dependence on temperature. Above the optimum temperature, microbial growth rate decreases and may lead to cellular lysis; furthermore, temperature effect analysis can assist in the determination of optimum conditions for cyanide biodegradation. However, increased biomass growth rate may not result in an increase in cyanide degradation rates, especially, if the microorganism is not substrate specific; a kinetic study for cyanide biodegradation and microbial growth can elucidate and verify this phenomenon.

Cape Peninsula
University of Technology

### 4.1.6 Statistical Model Analysis

The responses were subjected to an analysis using a Sequential Model Sum of Squares and a Lack of Fit tests to give a statistical model that suggested means and an adequate simulation using a quadratic model for cyanide degradation for a period of 72 hours after incubation. The responses were analysed using analysis of variance (ANOVA) to assess the significance of temperature (A) and pH (B) in the model (Table 4.2). A quadratic model was obtained (Eq. 4.1), generated by a multiple regression analysis of the cyanide degradation data.

$$Y = 239 + 23.6A + 38.09B - 49.87A^2 - 3.62B^2 + 3.25AB \qquad (Eq. 4.1)$$

Where $Y$ is the predicted response of F-CN biodegradation in terms of coded values.

A model reduction was carried out in order to exclude any insignificant model terms except terms supporting model hierarchy for improved fitness of the model, thus Equation 4.1, was reduced to:

$$Y = 239 + 23.6A + 38.09B - 49.87A^2 \qquad (Eq. 4.2)$$

**Table 4. 2: ANOVA for F-CN biodegradation Response Quadratic Model**

| Factors | Coeff. Estimate | DF | Standard Error | 95% CL Low | 95% CL High | F Value | Prob > F | Significance |
|---|---|---|---|---|---|---|---|---|
| Intercept | 239 | 1 | 10.03 | 215.27 | 262.73 | | 0.0029 | S |
| A | 23.6 | 1 | 8.69 | 3.05 | 44.50 | 7.37 | 0.0300 | S |
| B | 38.09 | 1 | 8.69 | 17.54 | 58.63 | 19.21 | 0.0032 | S |
| $A^2$ | -49.87 | 1 | 9.05 | -71.26 | -28.49 | 30.40 | 0.0009 | S |
| $B^2$ | -3.62 | 1 | 9.05 | -25.01 | 17.76 | 0.16 | 0.7005 | NS |
| AB | 3.25 | 1 | 12.29 | -25.81 | 32.31 | 0.07 | 0.7991 | NS |

S = Significant; NS = Not significant; CL = Confidence level; DF = Degree of freedom; "Prob.>F" less than 0.05 indicates model term is significant while values greater than 0.1 indicates model term is not significant; Std. Dev. = 24.58; R2 = 0.8907; Adj. R2 = 0.8127; Adeq. Precision = 10.341; C.V = 11.79

**Table 4. 3: Analysis of variance (Partial Sum of squares)**

| Source of Variation | Sum of squares | DF | Mean Square | F - value |
|---------------------|----------------|-----|-------------|-----------|
| Model | 34471 | 5 | 6894.35 | 11.41 |
| Residual | 4229.41 | 7 | 604.20 | |
| Lack of fit | 4229.41 | 3 | 1409.80 | 0.000 |
| Pure error | 0.000 | 4 | 0.000 | |
| Cor. Total | 38701.43 | 13 | | |
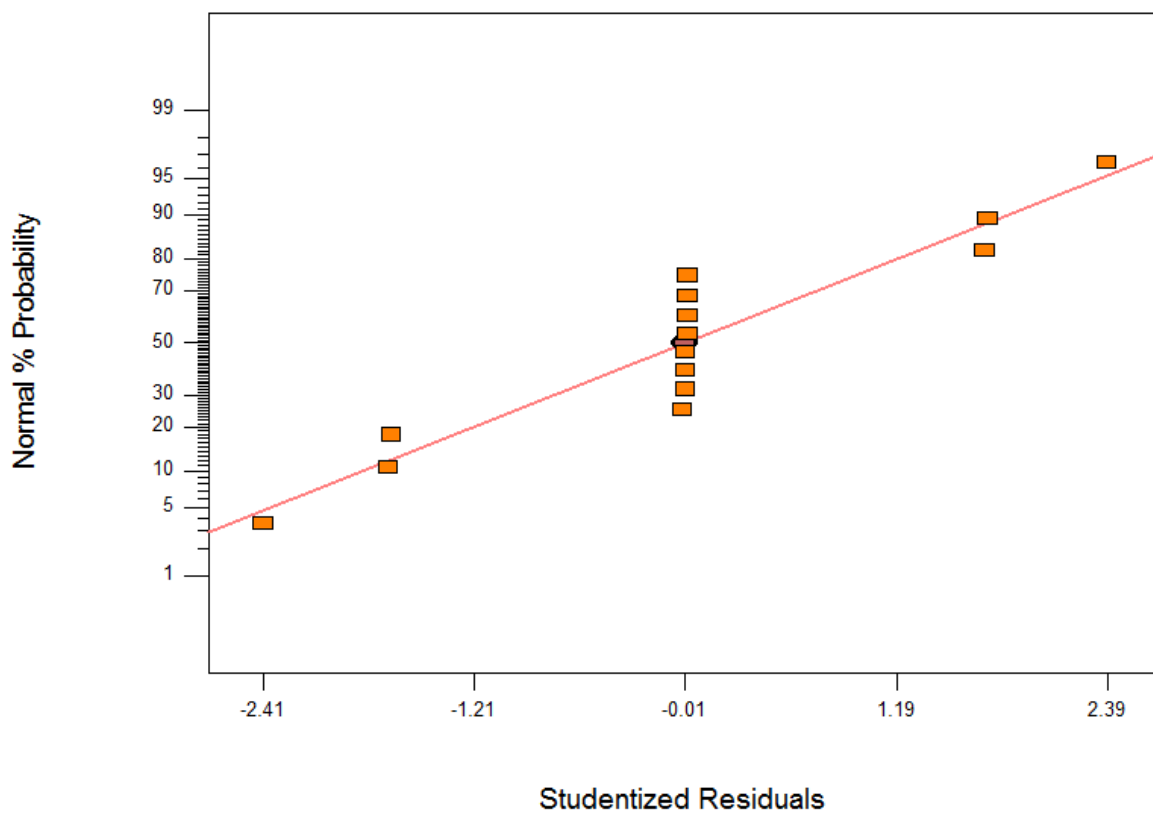


**Figure 4. 1: Normal probability plot of residual F-CN**

The Model F-value of 11.41 for the F-CN biodegradation was significant, indicating a 0.29% chance that a "Model F-Value" this large could occur due to noise for the quadratic model. Correlation coefficient, $R^2$ (0.8907) showed a high correlation of experimental data with predicted values. The $P$ values showed the level of interaction between variables with

temperature (A) having a significant impact than pH (B) on F-CN biodegradation. Statistically, an adequate precision of ratio greater than 4 is desirable for measuring a signal to noise ratio. The adequate precision of 10.341 observed in this study indicated an adequate signal that can be used to further navigate the design space. The coefficient of variance (CV) shows the degree of precision reliability of the experiments. The lower the CV value, the higher the experiment reliability. The CV of 11.79% emphasizes the precision and reliability of this model. The non-significant F-value for the Lack of Fit Test (Table 4.3) also justified the model suitability. Likewise, studentized residual plot shown in Figure 4.1 showed normality in the error term.

### 4.1.7 Graphical representation of the response model

The interaction between the independent variables was studied by plotting three dimensional (3-D) curves of the response against the variables. This allowed for the interpretation of experimental results and the determination of optimal conditions. Elliptical contour showed the interaction between the independent variables was sufficient and/or suitable while a circular contour indicated the variables are non-interactive ((Montgomery, 2008; Myers *et al.*, 2009)). Figure 4.2 a and b show 3-D plots and the corresponding contour plots for F-CN biodegradation. The plots show an increase in F-CN biodegradation as the pH and temperature increases until the temperature reaches approximately 24°C, where the F-CN biodegradation started decreasing. Figure 4.2c and d shows 3-D and contour plots for residual ammonium-nitrogen produced during F-CN biodegradation. The $NH_4^+$-N produced was observed to be reduced away from the centre point. Hence, either extreme conditions, i.e. low temperature and low pH or high temperature and high pH can favour minimal residual ammonium-nitrogen production. Figure 4.2e and f show plots of 3-D and contours for residual nitrate-nitrogen produced. Away from the centre point, the amount of nitrate produced also increased. Operating at either extreme conditions, it was observed to maximise nitrate production. In all cases, the response plots in Figure 4.2 show appreciable interaction between the output and input variables.
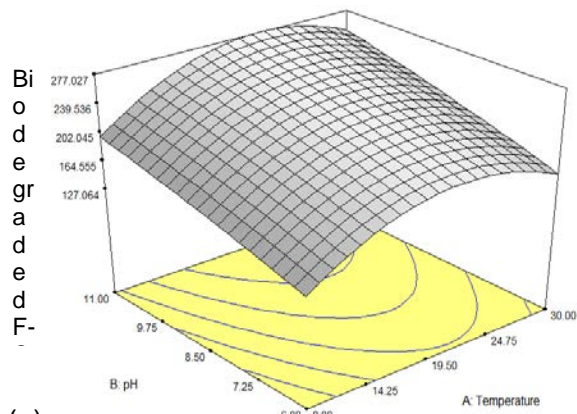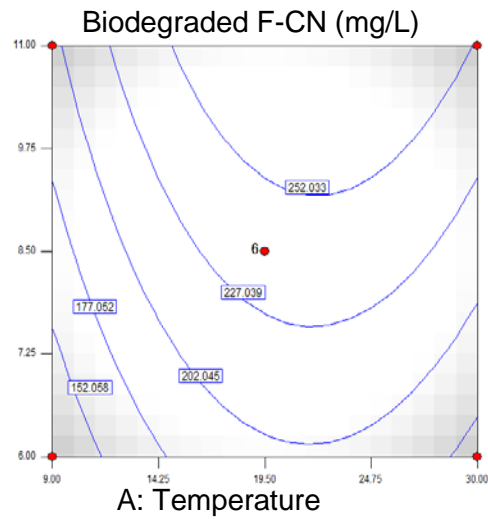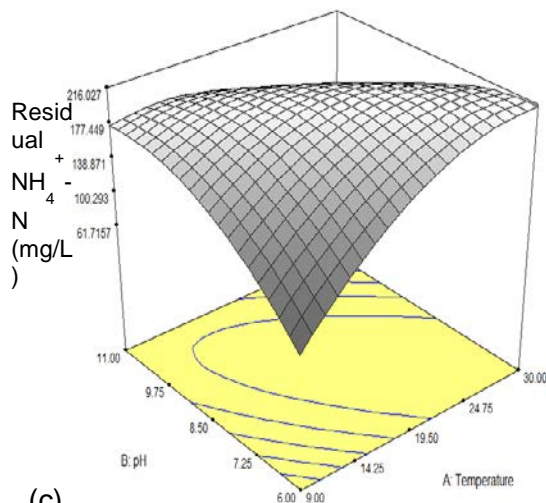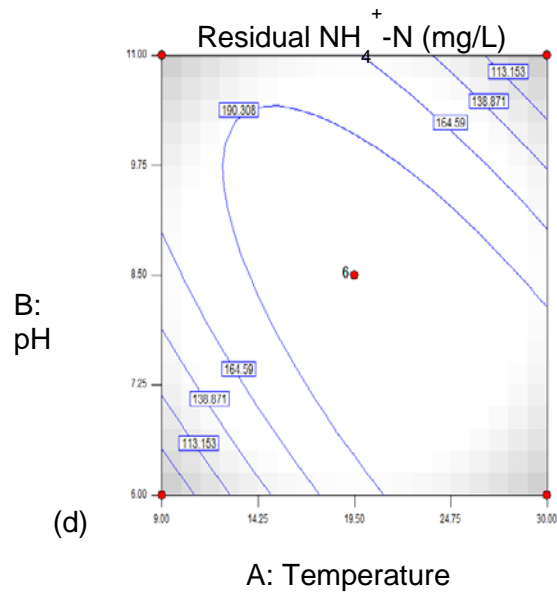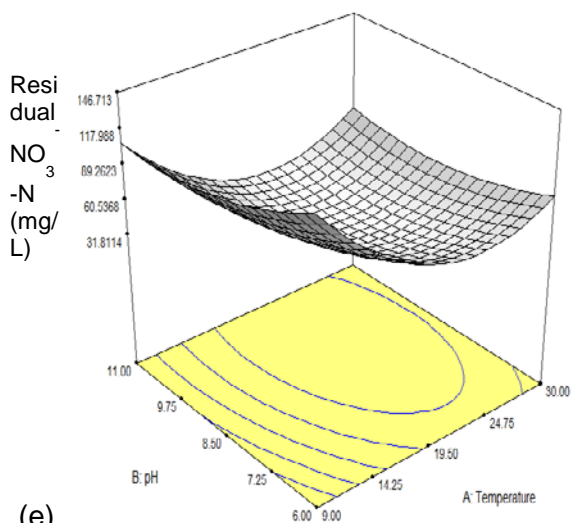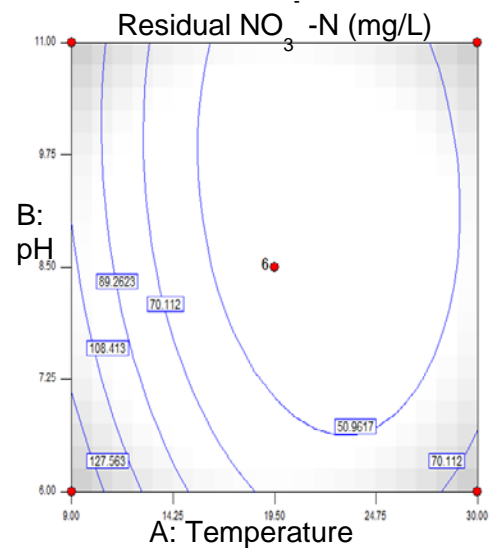
**Figure 4. 2: 3-D plots a, c, and e and contour plots b, d, and f showing interactive effects of independent variables on F-CN biodegradation and residual products**

51

### 4.1.8 F-CN biodegradation optimisation

The Design-Expert® software's numerical option with selected input factors was used to optimise the response in order to achieve peak performance. In a numerical optimisation, the desired goal for both input variables (temperature and pH) and responses is selected using a weight to emphasise the degree of importance of a single goal relative to the other. The numerical optimisation response can be set to maximise, minimise or target a value while the input variables can be set within range, target, none, minimise, or maximise.
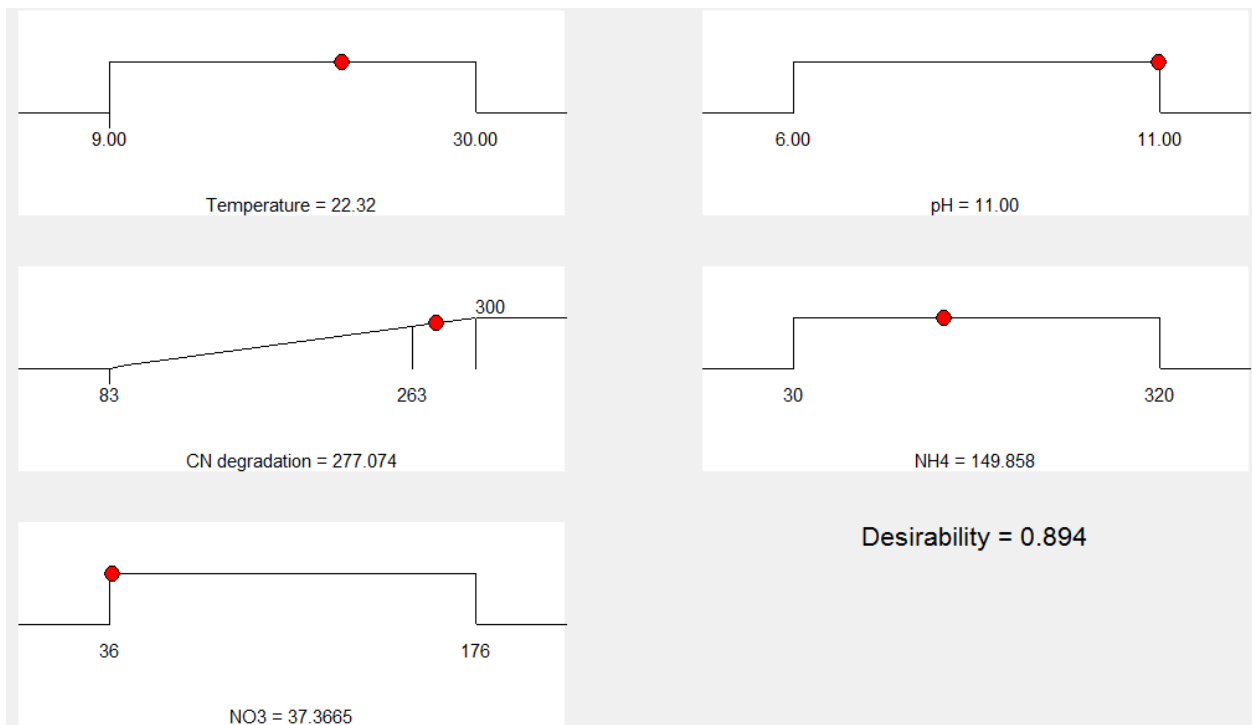


**Figure 4. 3: Desirability ramp for the numerical optimisation of F-CN biodegradation**

In this study, input variables, ammonium-nitrogen and nitrate-nitrogen were set within range; free cyanide biodegradation response was set to a maximum. The resultant output gave a list of solutions that match the set criteria from the least to the most desirable. The solution with the highest desirability from the ten options through which the numerical optimisation was selected –see Figure 4.3. The optimum condition for maximum cyanide biodegradation of 277 mg F-CN/L from an initial concentration of 500 mg F-CN/L over a period of 72 hours incubation was found at a temperature and pH of 22.32°C and 11, respectively. Previous studies by Pereira *et al.* (1996) and Campos *et al.* (2006) on the biodegradation of cyanide using *Fusarium oxysporum*, operated at a temperature ranging from 25 to 30°C and a pH of 8 using refined carbon source –

see Table 2.6, whereas in this study, *Beta vulgaris* waste was used as a carbon source in the presence of heavy metals for cyanide biodegradation.

### 4.1.9 Summary

Two independent variables (temperature and pH) assessed for modelling and optimisation played a vital role in free cyanide biodegradation. The quadratic model obtained provided suitable predictions for the variables evaluated with correlation coefficient of ($R^2$) 0.8907 being achieved. Also statistical model analysis showed that temperature was predominantly significant. From the numerical optimisation, an approximate maximum cyanide degradation of 277 mg F-CN/L at pH of 11 and temperature of 22°C in the presence of heavy metals can be achieved within 72 hours.

### 4.2 Cyanide biodegradation Kinetics

### 4.2.1 Introduction

There was a need to determine the biodegradation rate kinetics for cyanide cultures after knowing the optimum operating conditions. Discharged wastewater often contains low concentration of cyanide complexes which result from the incomplete degradation of cyanide. Ordinary Differential Equation (ODE) solver, (version 5.0) was used to analyse the degradation rate dynamics and variations over time. Two different temperatures, 22°C (optimum temperature) and 5°C (temperature representing cold/winter conditions) at the same pH of 11 (previously determined to be the optimum pH) for cyanide concentrations of 100 mg F-CN/L, 200 mg F-CN/L and 300 mg F-CN/L, were studied.

### 4.2.2 Aim

The aims were to:

- determine the rate of degradation of free cyanide;
- use the ODE to simulate the free cyanide biodegradation kinetics; and
- use the Arrhenius model to investigate the effect of temperature on the free cyanide biodegradation.

### 4.2.3 Free cyanide degradation kinetics

The free cyanide degradation efficiency of *Fusarium oxysporum* was studied at 100, 200, and 300 mg F-CN/L. At a temperature of 22°C, the *Fusarium oxysporum* culture showed removal efficiency of 77%, 58% and 62% for cultures containing 100, 200, and 300 mg F-CN/L respectively –see Figure 4.4a. Statistical Package (SPSS version 22) software was used to analyse the removal efficiency which showed that removal efficiency in cultures containing cyanide concentration of 200 and 300 mg F-CN/L has correlation coefficients 0.8943 and 0.9017, respectively, and were statistically the same at significant level of 0.01. This showed that the *Fusarium oxysporum* was more efficient at lower cyanide concentration as expected. The microorganism was able to degrade 65 mg F-CN/L, 108 mg F-CN/L and 173 mg F-CN/L of free cyanide from 100, 200 and 300 mg F-CN/L of cyanide solutions, respectively, within 144 hours. Free cyanide loss due to volitilisation was about 2.7%, 2.2% and 3.2% for 100, 200 and 300 mg F-CN/L –see Figure 4.4b. This is an improvement on previous study by Cabuk *et al.* (2006) when white rot fungus, *Trametes versicolor* removal efficiency of F-CN was less than 30%. Although, Campos *et al.* (2006) and Ezzi *et al.* (1995) reported higher removal efficiency of 90%, other microorganisms were used to support *Fusarium oxysporum* and the incubation period was above 720 hours as reported by Ezzi *et al.* (1995). The residual ammonium-nitrogen concentration fluctuated between 70 to 210 mg $NH_4^+$-N/L (Figure 4.4c) throughout the experiments. High residual ammonium-nitrogen concentration in cultures inhibited cyanide degradation due to the microorganism preference for it as a nutritional source in the presence of cyanide. The residual nitrate-nitrogen formed was between 61 mg $NO_3^-$-N/L and 102 mg $NO_3^-$-N/L over the period under observation (Figure 4.4d).


At a cold temperature (5°C), the free cyanide removal efficiency was 51% with residual 39 mg F-CN/L for 100 mg F-CN/L, 43% with residual 104 mg F-CN/L for 200 mg F-CN/L and 44% with residual 155 mg F-CN/L for 300 mg F-CN/L cultures over an observation period of 144 hours. Volatilisation of free cyanide was about 3.3%, 2.8% and 4.2% for 100, 200 and 300 mg F-CN/L. The cold temperature affected the activity of the microorganism. The high amount of residual ammonium-nitrogen (up to 50 mg $NH_4^+$-N/L) and nitrate-nitrogen (up to 140 mg $NO_3^-$-N/L) showed the impact of the temperature on cyanide biodegradation – see Figure 4.5.

(a)

(b)

(c)

(d)

**Figure 4. 4: (a) Relationship between cyanide biodegradation removal efficiency and growth of *Fusarium oxysporum* at 100, 200, and 300 mg F-CN/L (b) Cyanide biodegradation in the culture (c) Residual ammonium concentration (d) Residual nitrate concentration during cyanide biodegradation – at temperature 22°C**

Cape Peninsula
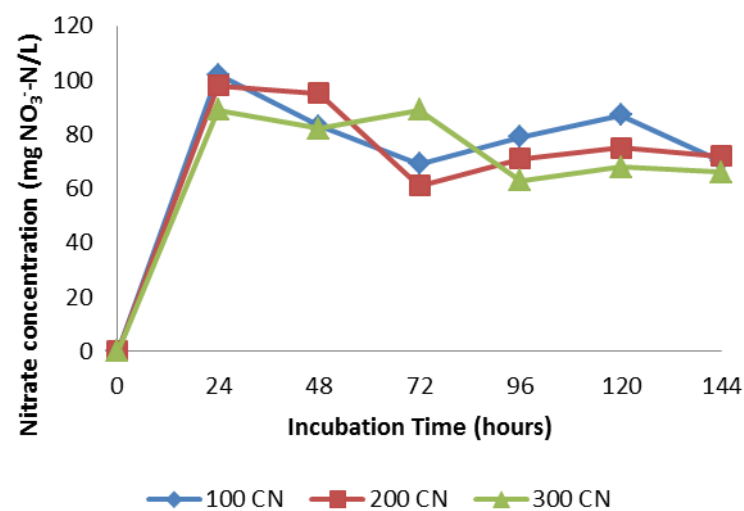University of Technology
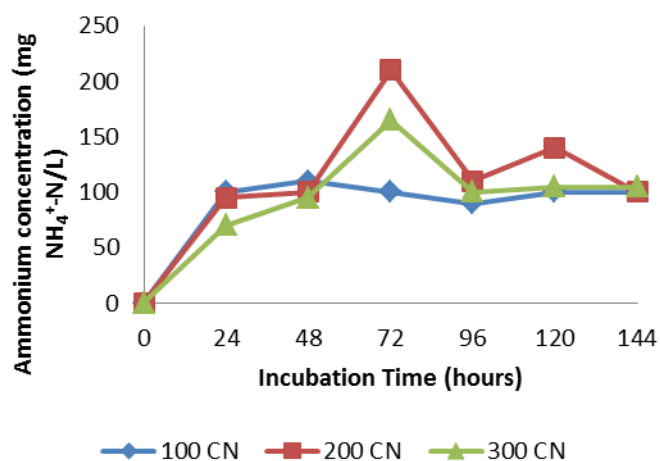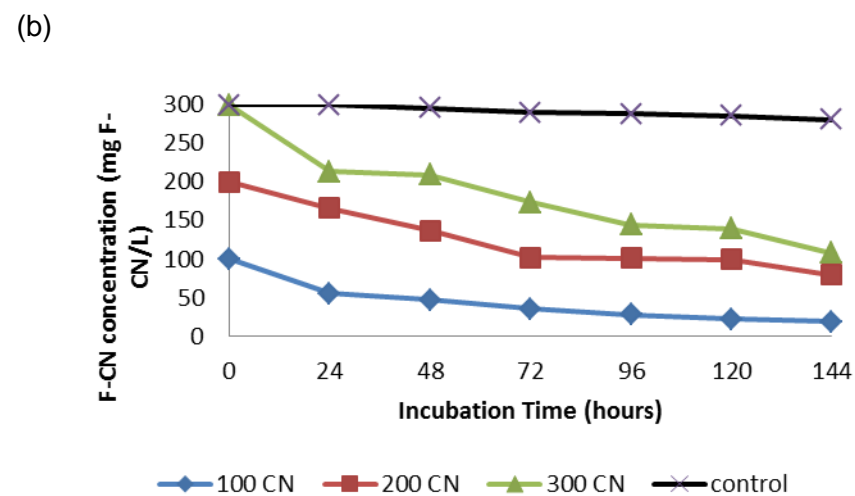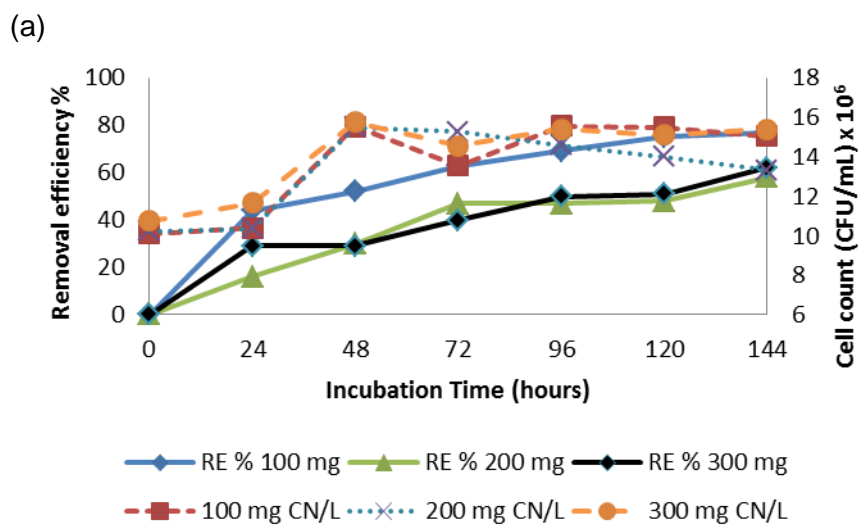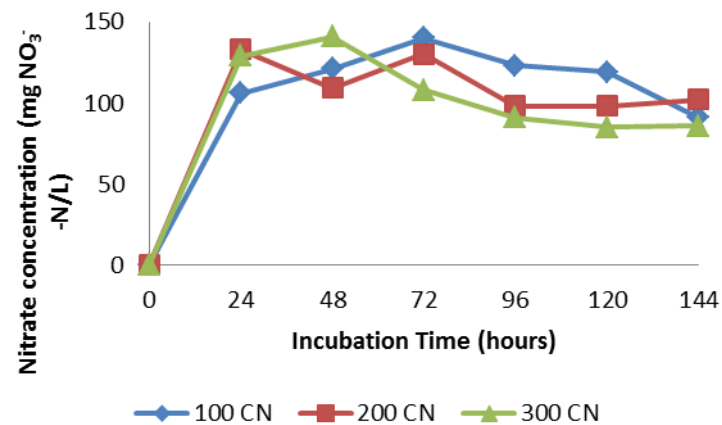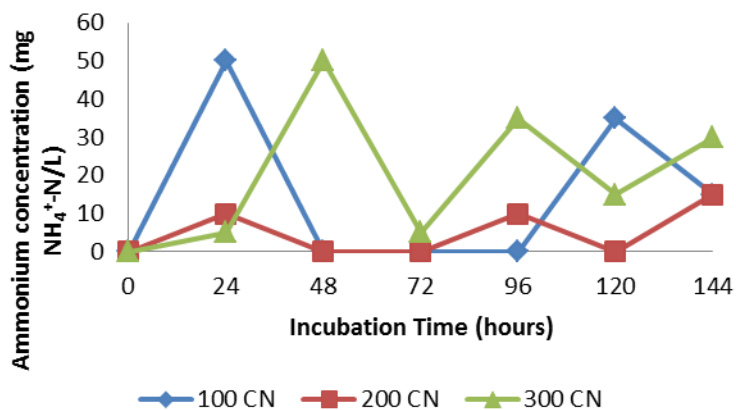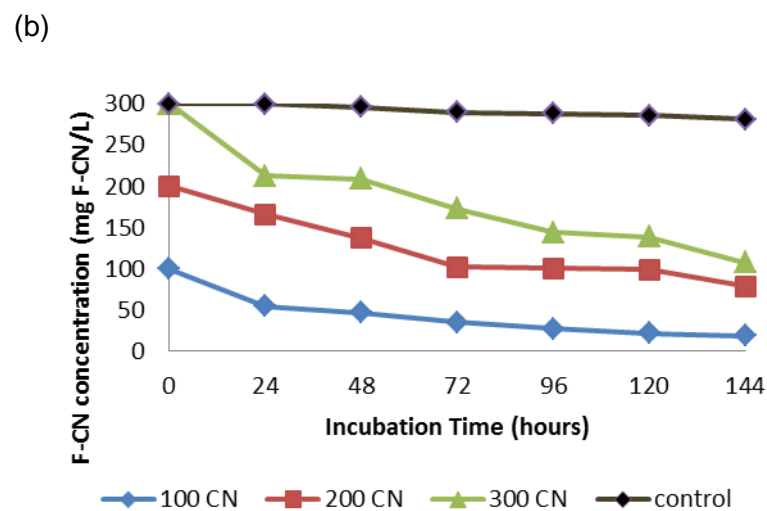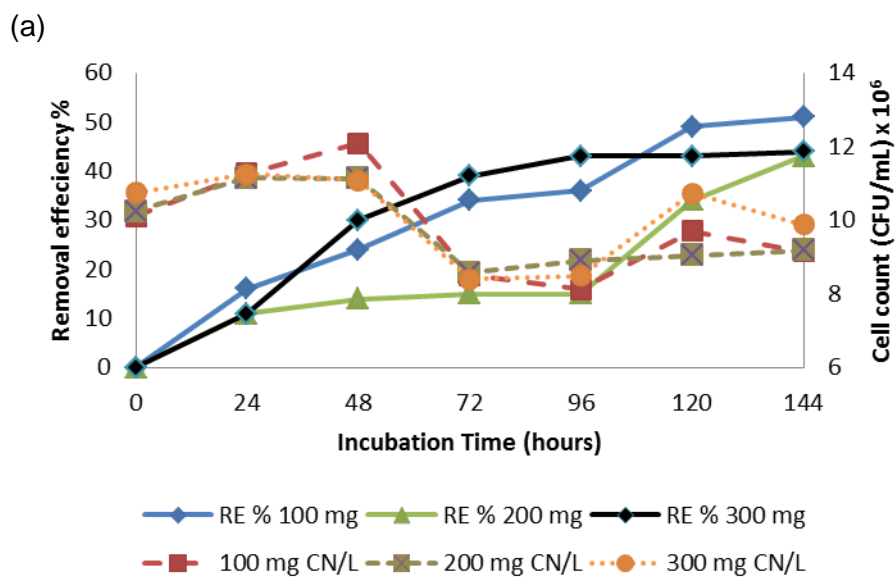
(a)

(b)

(c)

(d)

**Figure 4. 5: (a) Relationship between cyanide biodegradation removal efficiency and growth of *Fusarium oxysporum* at 100, 200, and 300 mg F-CN/L (b) Cyanide biodegradation in the culture (c) Residual ammonium concentration (d) Residual nitrate concentration during cyanide biodegradation – at temperature 5°C**

Cape Peninsula
University of Technology

### 4.2.4 Free cyanide degradation rate model

The rate of degradation was estimated over 144 hours assuming first order kinetics using Equation 3.9 which was used,( $\frac{d[CN]}{dt} = -k[CN]$ ) in an Ordinary Differential Equation (ODE) solver to model the cyanide biodegradation kinetics. The rate of cyanide degradation was higher at F-CN concentration of 100 mg F-CN/L, it reduces as the F-CN concentration increases to 200 mg F-CN/L and then peak up at 300 mg F-CN/L. On the other hand, as the temperature was reduced, the rate of cyanide degradation also decreased (see Table 4.4).

**Table 4. 4: Ordinary Differential Equation solver input parameters**

| Rate equation: $\frac{d[CN]}{dt} = -k[CN]$ | | |
|---|---|---|
| **Time: 0 h < t < 144 h** | | |
| C(0) = [CN⁻]$_{t=0}$ | Temperature = 22°C | Temperature = 5°C |
| 100 | $k = 0.0115$ h$^{-1}$ | $k = 0.0065$ h$^{-1}$ |
| 200 | $k = 0.0065$ h$^{-1}$ | $k = 0.0045$ h$^{-1}$ |
| 300 | $k = 0.0071$ h$^{-1}$ | $k = 0.0046$ h$^{-1}$ |

The high degradation rate observed may be attributed to the sufficient enzyme activity aided by suitable bioreactor conditions and the utilisation of *Beta vulgaris* which contains soluble sugars, minerals and proteins to support enzyme production. Earlier reports have shown that substrates such as *Beta vulgaris* have a large content in pectin, reducing sugars, cellulose and protein (Mulligan, 2005; Anuradha *et al.*, 2010; Amodu *et al.*, 2014). Anuradha *et al.* (2010) showed that *Fusarium oxysporum* is capable of multienzyme production when grown on *Beta vulgaris*, orange peel, carrot peel and pineapple peel, hence, the performance of this organism in high concentration of cyanide containing wastewater. Figure 4.6, shows that the accuracy between the modelled and experimental values was high, especially at a higher temperature. The correlation of coefficient R$^2$ for the entire ODE model was 0.9567 (100 mg F-CN/L), 0.9856 (200 mg F-CN/L) and 0.9828 (300 mg F-CN/L) at 22°C while 0.9856 (100 mg F-CN/L), 0.9930 (200 mg F-CN/L) and 0.9927 (300 mg F-CN/L) at 5°C which implies that the model developed was suitable.

Cape Peninsula
University of Technology
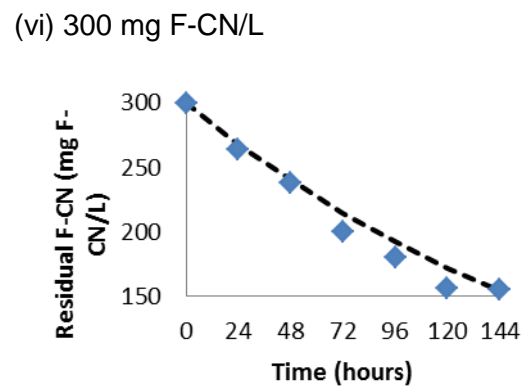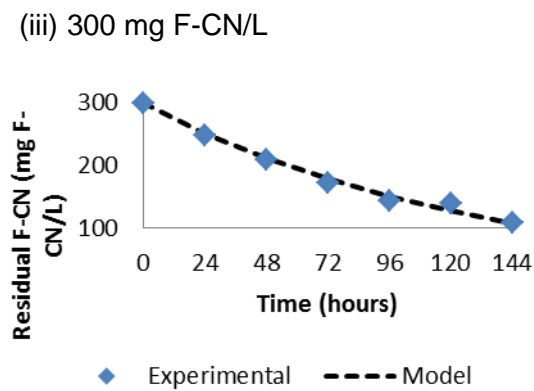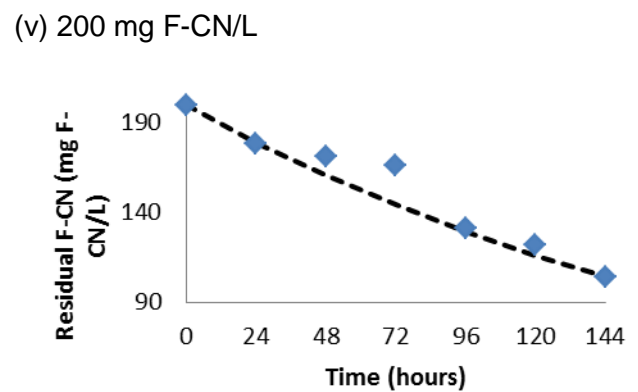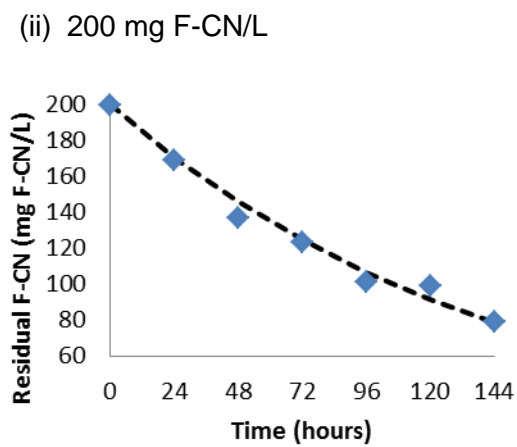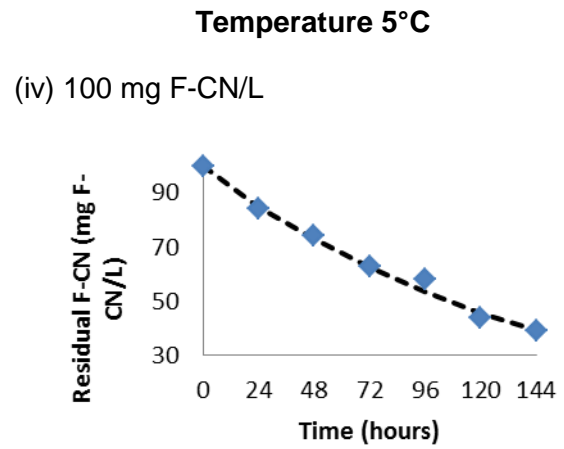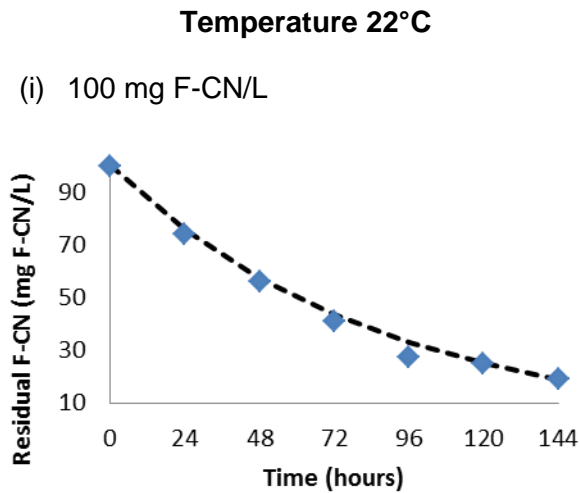
**Figure 4. 6: Comparison between experimental and modelled bioremediation kinetics at temperatures 22°C (i, ii and iii) and 5°C (iv, v and vi)**

Cape Peninsula
University of Technology

### 4.2.5. Effect of temperature on cyanide biodegradation

The effect of temperature on the rate of cyanide biodegradation was very pronounced at 22°C. At 5°C, the biodegradation process was slow. The activation energy $E$, was calculated for KCN solution used in this study by applying the Arrhenius equation 3.15.

**Table 4. 5: Energy of Activation for F-CN degradation**

| F-CN concentration (mg F-CN/L) | Activation Energy $E$ (KJ/mol) | Frequency factor $A$ ($h^{-1}$) |
|---|---|---|
| 100 | 19.6 | 34.7 |
| 200 | 12.7 | 1.14 |
| 300 | 14.9 | 3.15 |

The energy of activation was positive thus the increase in rate constants with increasing temperature as shown in Table 4.4. The value of $E$ was higher at 100 mg F-CN/L due to the reduced inhibitory effect of the cyanide as indicated by the frequency factor in Table 4.5. Frequency factor denotes the level of interaction between the microbial system and cyanide which was high at lower cyanide concentration. SPSS version 22 was used to ascertain the significance of frequency factor in cultures containing cyanide concentration of 200 and 300 mg F-CN/L, it was found to be significantly tsimilar. Although the activation energy was lower at 200 mg F-CN/L, but this did not translate to an increased rate of reaction, this could be due to numerous metabolic activities being reduced and/or that were impeded at this cyanide concentration.

### 4.2.6 Summary

Biodegradation of cyanide was successful in all the cultures with subsequent production of ammonium-nitrogen and nitrate-nitrogen. However, ammonium-nitrogen inhibited the cyanide biodegradation while in certain instances it was not detected, yet nitrate–nitrogen was produced -an indication that the microorganism sometimes either produced small amount of ammonium-nitrogen which was quickly consumed or converted to nitrate-nitrogen under cold temperature (Figure 4.5c). A large quantity of residual nitrate-nitrogen was produced in all cases under observation; which the organism can metabolise although inhibiting cyanide degradation. The rate of degradation of F-CN in the presence of heavy metals was higher at a low concentration

Cape Peninsula
University of Technology

of F-CN due to the limited amount of ammonium-nitrogen and nitrate-nitrogen produced athigher temperature combined with an assumed multienzyme production activity for the *Fusarium oxysporum* culture. Increased temperature was found to be responsible for the high rate of cyanide degradation.

### 4.3 *Fusarium oxysporum* growth kinetics

### 4.3.1 Introduction

The biodegradation of cyanide occurs as the microorganism feeds on the cyanide as a nutritional source. The success of the process was dependent on the cyanide assimilation pathway of the organism and the mechanism used for cyanide tolerance. A majority of microorganisms that feed on cyanide grow at pH 7-9, thereby a large amount of cyanide exist as cyanide ion ($CN^-$) (Dash *et al.*, 2009b). In this study, *Fusarium oxysporum* was grown on *Beta vulgaris* at pH exceeding 9, i.e. pH = 11 and temperature of 22°C and 5°C. For cyanide degradation, microbial growth and activity play an important role, thus the need to quantify *Fusarium oxysporum* growth kinetics under these conditions.

### 4.3.2 Aims

The aims were to:

- study *Fusarium oxysporum* growth kinetics using the Monod's growth kinetic model; and
- investigate the effect of temperature on *Fusarium oxysporum* specific growth rate constant using the Arrhenius model (Equation 4.2).

### 4.3.3 Quantification of *Fusarium oxysporum* population growth

The cell concentration in the medium varied depending on the amount of cyanide concentration in the culture. From Figures 4.7 and 4.8, it was clear that the cyanide concentration inhibited the fungus microbial growth. Under optimum temperature of 22°C, the maximum cell concentration in 100, 200 and 300 mg F-CN/L were 1.557 x $10^7$ CFU/mL, 1.548 x $10^7$ CFU/mL and 1.575 x$10^7$ CFU/mL, respectively –see Figure 4.7. The control experiment with no cyanide in the culture has maximum cell concentration of 2.317 x$10^7$ CFU/mL. The maximum growth rate observed was 0.0166 $h^{-1}$, 0.0168 $h^{-1}$ and 0.012 $h^{-1}$ for 100, 200, and 300 mg F-CN/L, respectively. The lag

phase at a temperature of 22°C was 24 hours both in non-cyanide and cyanide containing cultures; however, the exponential phase lasted for 48 hours in non-cyanide cultures but with accelerated growth that took 24 hours in all medium containing cyanide, achieving different maximum cell concentrations.

In addition, under temperature of 22°C, a decelerated growth was noticed in non-cyanide culture between 72 h and 96 h prior to the death phase; observing minimal cellular growth deceleration in all medium containing cyanide preceding intermittent death and a minimal cell growth observed thereafter (Figure 4.7). This could be as a result of microorganism changing nutritional sources from cyanide to either ammonium-nitrogen or nitrate-nitrogen and the depletion of other media nutrients from the *Beta vulgaris*. The free cyanide saturation constants ($K_s$) of 86.5, 190.7 and 150 mg F-CN/L for initial cyanide concentrations of 100, 200 and 300 mg F-CN/L, respectively, was observed. This showed that the microorganism had a higher affinity for the cyanide at concentration of 200 mg F-CN/L which also reflected in the high growth rate and minimal doubling time – see Table 4.6.
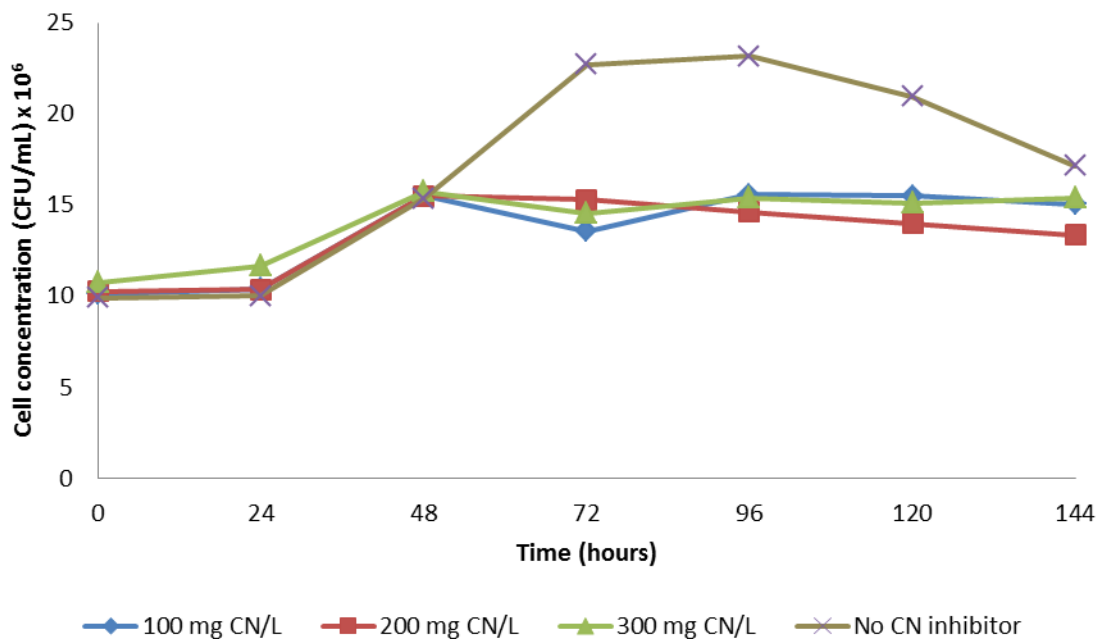


**Figure 4. 7: *Fusarium oxysporum* growth at temperature 22°C**
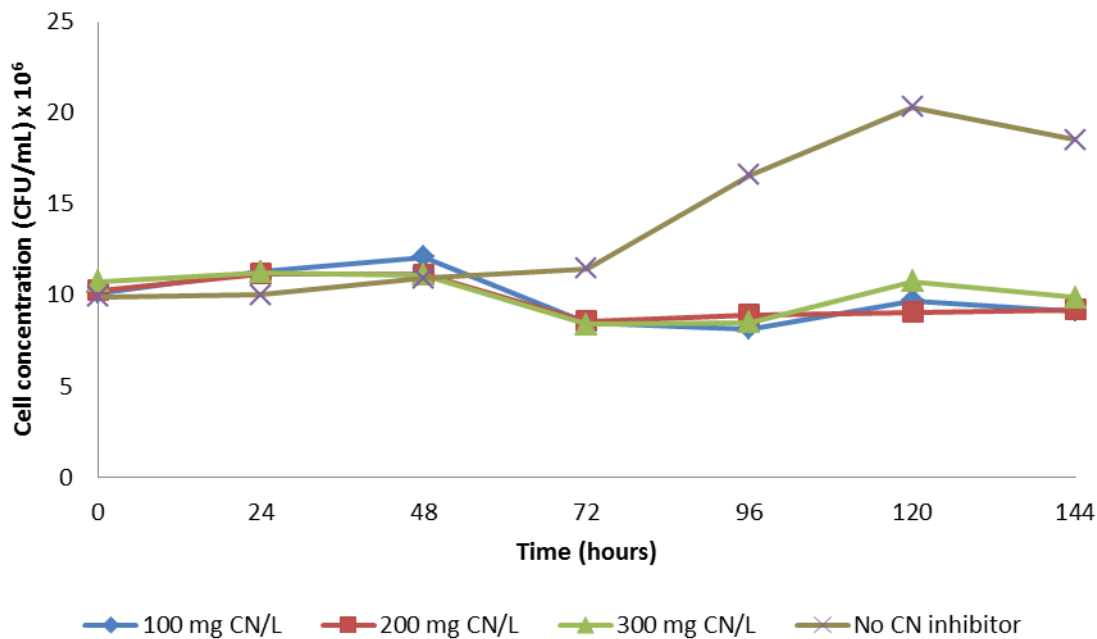
**Figure 4. 8:** *Fusarium oxysporum* growth at temperature 5°C

**Table 4. 6:  Growth kinetics parameters of *Fusarium oxysporum***

| F-CN concentration (mg F-CN/L) | Temperature 5°C | | | Temperature 22°C | | |
|---|---|---|---|---|---|---|
| | $\mu_m$ (h$^{-1}$) | $K_s$ (mg F-CN/L) | $t_d$ (h) | $\mu_m$ (h$^{-1}$) | $K_s$ (mg F-CN/L) | $t_d$ (h) |
| 100 | 0.0045 | 21.6 | 154 | 0.0166 | 86.5 | 42 |
| 200 | 0.0035 | ≤ 100 | 198 | 0.0168 | 190.7 | 41 |
| 300 | 0.0019 | ≤ 150 | 365 | 0.0120 | 150 | 58 |
| No F-CN | 0.0154 | - | 45 | 0.0178 | - | 39 |

Under cold temperature (5°C), observations (Figure 4.8) showed that microbial growth was slow. The maximum cell concentration for 100, 200 and 300 mg F-CN/L were 1.208 x10$^7$ CFU/mL, 1.115 x10$^7$ CFU/mL and 1.125 x 10$^7$ CFU/mL, respectively, while for non-cyanide was 2.032 x10$^7$ CFU/mL. The maximum growth rate of 0.0045 h$^{-1}$, 0.0035 h$^{-1}$ and 0.0019 h$^{-1}$ for 100 mg F-CN/L, 200 mg F-CN/L and 300 mg F-CN/L, respectively, was observed. As a result of the reduction in growth rates at the cold temperature, the microbial doubling time was considerably

higher – see Table 4.6. The free cyanide saturation constants ($K_s$) of 21.6, $\leq$ 100, and $\leq$ 150 mg F-CN/L for initial cyanide concentrations of 100, 200 and 300 mg F-CN/L, respectively, was observed. For concentrations 200 and 300 mg F-CN/L, the microbial growth rate was similar to the maximum growth rate; hence, the Blackman model equation was used to estimate the saturation constant i.e. $2\,K_s \leq S\ when\ \mu = \mu_m$.

Furthermore, the non-cyanide culture lag phase was longer (72 hours), before reaching the exponential growth phase which lasted for 48 hours before the death phase. However, the cell growth pattern was generally irregular in cyanide containing media probably due to the harsh environmental conditions. After 24 hours of incubation, cell death was observed in 200 and 300 mg F-CN/L cultures. Only the 100 mg F-CN/L medium, showed accelerated growth within the first 48 hours of incubation with maximum growth rate of 0.0045 h$^{-1}$. Cold temperature has been reported to hinder microbial growth of microorganisms used for bioremediation thus inhibiting contaminant removal processes (Zilouei *et al.*, 2006). Overall, the microbial growth depended on the initial free cyanide concentration as reflected in the low doubling time for medium without free cyanide (Table 4.6).

### 4.3.4 Effect of temperature on *Fusarium oxysporum* growth

Temperature is known to affect the configuration of microbial cell components. Generally, for every 10°C rise in temperature, there is a two-fold increase in the specific growth rate (Parolini and Carcano, 2010). In this study, Arrhenius equation 3.11 was applied to give:

$$\mu = Ae^{-E/RT} \qquad\qquad (Eq.\,4.1)$$

and for two different temperatures with the necessary adjustment, Equation 3.15 can be written for microbial growth as;

$$\ln\left(\frac{\mu_1}{\mu_2}\right) = \left(\frac{1}{T_2} - \frac{1}{T_1}\right)\frac{E}{R} \qquad\qquad (Eq.\,4.2)$$

The activation energy, $E$ for microbial cell growth in cyanide was calculated using Equation 4.2.

**Table 4. 7: Activation energy for *Fusarium oxysporum* cultures under different cyanide concentration**

| F-CN concentration (mg F-CN/L) | Activation energy $E$ (KJ/mol) |
|:---:|:---:|
| 100 | 44.9 |
| 200 | 54.0 |
| 300 | 63.5 |
| No F-CN | 4.9 |

The positive values of activation energy obtained (Table 4.7) justifies the increase in the specific growth rate with temperature increase from 5°C to 22°C observed in Table 4.6. The value of $E$ increased with an increase in the concentration of free cyanide. The highest value of activation energy (63.5 KJ/mol) recorded in 300 mg F-CN/L cultures explains the reason for low growth rates observed resulting from impaired microbial activity. During cellular respiration, microorganism generates energy in form of adenosine triphosphate (ATP) that is used to breakdown nutrients into smaller usable components. This energy is made available by the hydrolysis of ATP in water and must be greater than the activation energy for growth to take place (Parolini *et al.*, 2010). In this study, activation energy for *Fusarium oxysporum* growth in Table 4.7 is greater than activation energy for F-CN degradation in Table 4.5; this explained the reason for the appreciable growth rate and the subsequent F-CN degradation observed.

### 4.3.5 Summary

The *Fusarium oxysporum* grew successfully even under a high concentration of cyanide. The maximum growth of the microorganism was observed at concentration 300 mg F-CN/L, temperature 22°C to be 1.575 x 10$^7$ CFU/mL over a period of 144 hours but did not translate to maximum specific growth rate; this was observed for cultures having a concentration of 200 mg F-CN/L, which gave the highest specific growth rate of 0.0168 h$^{-1}$ with doubling time of 41 hours under the same temperature. The *Fusarium oxysporum* culture was functionally inhibited especially under a cold temperature; perhaps shifting metabolic mechanisms for survival depending on the available nutrient (cyanide, ammonium-nitrogen or nitrate-nitrogen) source. The microorganism degradation potential could be enhance by acclimatisation and/or direct evolution to improve the functionality of the microorganism under low temperature and high

Cape Peninsula
University of Technology

cyanide concentration. The impact of low temperature was evident on the microbial growth, as very low growth rate was observed under cold temperature. For the 17°C rise in temperature (5°C to 22°C) of this study, the specific growth rate increased approximately by 4-fold, 5-fold and 6-fold in 100 mg F-CN/L, 200 mg F-CN/L and 300 mg F-CN/L respectively, an indication that this particular isolated *Fusarium oxysporum* from cyanide containing pesticides will most likely work better in process operated at higher temperature.

# CHAPTER 5

# CONCLUSIONS AND RECOMMENDATIONS

Cape Peninsula
University of Technology

# CHAPTER 5
# CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Conclusion

For a century, cyanide has been a preferred reagent in the extraction processes in the mineral processing industry thus its presence in wastewater is inevitable. Cyanide is a potential threat to both environment and aquatic life in general. Cyanide in wastewater converts to hydrogen cyanide in tailing pond and volatilise under increasing acidic conditions. Hence, cyanide containing wastewater must be treated or the cyanide be reduced to an acceptable standard before being discharged to the environment. Several biological treatments of cyanide have been reported but very few studies focused on application of agro-waste as a carbon/energy source in the biodegradation of cyanide. In this study, biodegradation of cyanide in the presence of metals (arsenic, copper, lead, iron and zinc) using *Fusarium oxysporum*, grown on *Beta vulgaris* extract as a sole carbon and or energy source was studied. The *Fusarium oxysporum* was isolated at the Bioresource Engineering Research Group (BioERG), at the Cape Peninsula University of Technology, Cape Town.

The impact of two independent variables namely; temperature and pH were studied using a response surface methodology (RSM). The use of *Beta vulgaris* with wastewater containing *Fusarium oxysporum* as performed in this study enhanced the degradation of cyanide. This has shown that a large quantity of agricultural waste been generated annually can be utilised as a nutrient source for microbial growth in bioremediation studies. Using a central composite design, the response obtained from the statistical model showed that temperature was a more significant factor during the biodegradation of cyanide with correlation coefficient ($R^2$) of 89% being achieved. The residual ammonium-nitrogen and nitrate-nitrogen produced was observed to be high playing a major role in inhibiting the cyanide biodegradation process. The optimum operating condition using the numerical optimisation option was found to be a temperature of 22°C and a pH of 11 with a maximum cyanide degradation of 277 mg F-CN/L from an initial F-CN concentration of 500 mg F-CN/L in the presence of heavy metals. This was achieved within 72 hrs.

Based on the optimal conditions obtained, a temperature of 22°C and simulating winter conditions (temperature of 5°C), a batch study of cyanide biodegradation kinetics and *Fusarium*

*oxysporum* growth kinetics was studied at cyanide concentrations of 100, 200 and 300 F-CN/L over a period of 144 hours. The degradation efficiency of free cyanide was higher at the optimum temperature (22°C); however, this led to a higher concentration of residual ammonium-nitrogen and nitrate-nitrogen formed especially at elevated concentrations of free cyanide. Although the residual by-products can serve as a nitrogen source for the fungus, there was an indication of incomplete metabolism. This can be enhanced by changing the operating conditions so that the microbial system can handle nitrification and denitrification of residual by-products after cyanide biodegradation.

The major driving force of cyanide biodegradation rate was found to be temperature as shown by the estimated rate of degradation of cyanide. The ordinary differential equation (ODE) model used to describe the cyanide removal rate was determined within a 95% confidence level of biodegradation rate. The means and standard deviations for $k$ values at 5°C and 22°C were: 0.0052 (± 0.0011) $h^{-1}$ and 0.0084 (± 0.0027) $h^{-1}$, respectively. The cyanide degradation rates varied with different cyanide concentrations. The application of the Arrhenius model equation fully described the estimation of the energy of activation for cyanide biodegradation. The activation energy showed that a minimum of 12.7 kJ/mol was needed for a mole of free cyanide to be degraded; however, a minimum of 44.9 kJ/mol was needed for microbial growth. Therefore, free cyanide degradation became the rate limiting step. The means and standard deviations of microbial growth rate at 5°C and 22°C were: 0.0033 (± 0.0013) $h^{-1}$ and 0.0151 (± 0.0027) $h^{-1}$, respectively. The growth rates varied with different free cyanide concentration and increased with temperature. The microbial growth kinetics could be modelled sufficiently with the Monod's growth kinetic model.

The alkaline operating conditions with minimal modifications in microbial growth temperature, resulted in the successful biodegradation of free cyanide in the presence of heavy metals. This process is environmentally friendly and can be improved for the holistic modelling and design of cyanide wastewater treatment plant before the water be discharged or re-used.

## 5.2 Recommendations

The following are recommendations for future research:

Cape Peninsula
University of Technology

- A model for continuous biodegradation of cyanide containing wastewater including microbial growth kinetics for such a process,

- A study on biodegradation of cyanide complexes with the biosorption of heavy metals in a continuous system,

- A model that can handle both cyanide biodegradation and subsequent nitrification and denitrification in cyanide containing wastewater,

- A study to assess the impact of UV on cyanide biodegradation, microbial growth, nitrification and denitrification in cyanide containing wastewater,

- A scale-up design for applicability and economic feasibility on an industrial scale could be investigated, and

- The elucidation of biocatalysis reactions and the type of enzymes produced by the *Fusarium oxysporum* used in this study.

Cape Peninsula
University of Technology

# REFERENCES

# REFERENCES

Acheampong, M., Paksirajan, K. and Lens, P. L. 2013. Assessment of the effluent quality from a gold mining industry in Ghana. *Environmental Science and Pollution Research*, 20(6): 3799-3811.

Acheampong, M. A., Meulepas, R. J. W. and Lens, P. N. L. 2010. Removal of heavy metals and cyanide from gold mine wastewater. *Journal of Chemical Technology & Biotechnology*, 85(5): 590-613.

Adams, D., Komen, J. and Pickett, T. 2001. Biological cyanide degradation. *Cyanide: Social, Industrial and Economic Aspects. The Metals Society, Warrendale, PA*: 203-213.

Adjei, M. D. and Ohta, Y. 2000. Factors affecting the biodegradation of cyanide by Burkholderia cepacia strain C-3. *Journal of Bioscience and Bioengineering*, 89(3): 274-277.

Akcil, A. and Mudder, T. 2003. Microbial destruction of cyanide wastes in gold mining: process review. *Biotechnology Letters*, 25(6): 445-450.

Amodu, O. S., Ntwampe, S. K. and Ojumu, T. V. 2014. Emulsification of Hydrocarbons by Biosurfactant: Exclusive Use of Agrowaste. *BioResources*, 9(2): 3508-3525.

Anderson, M. J. and Whitcomb, P. J. 2005. RSM simplified: optimizing processes using response surface methods for design of experiments, Productivity press.

Anuradha, K., Padma, P. N., Venkateshwar, S. and Reddy, G. 2010. Fungal isolates from natural pectic substrates for polygalacturonase and multienzyme production. *Indian Journal of Microbiology*, 50(3): 339-344.

Atkinson, A. 1975. Bacterial cyanide detoxification. *Biotechnology and Bioengineering*, 17(3): 457-460.

Babel, S. and Kurniawan, T. A. 2004. Cr(VI) removal from synthetic wastewater using coconut shell charcoal and commercial activated carbon modified with oxidizing agents and/or chitosan. *Chemosphere*, 54(7): 951-967.

Babu, G. R. V., Wolfram, J. and Chapatwala, K. 1992. Conversion of sodium cyanide to carbon dioxide and ammonia by immobilized cells of *Pseudomonas putida*. *Journal of Industrial Microbiology*, 9(3-4): 235-238.

Barclay, M., Tett, V. A. and Knowles, C. J. 1998. Metabolism and enzymology of cyanide/metallocyanide biodegradation by *Fusarium solani* under neutral and acidic conditions. *Enzyme and Microbial Technology*, 23(5): 321-330.

Cabuk, A., Unal, A. and Kolankaya, N. 2006. Biodegradation of cyanide by a white rot fungus, *Trametes versicolor. Biotechnology Letters*, 28(16): 1313-1317.

Campos, M. G., Pereira, P. and Roseiro, J. C. 2006. Packed-bed reactor for the integrated biodegradation of cyanide and formamide by immobilised *Fusarium oxysporum* CCMI 876 and *Methylobacterium sp.* RXM CCMI 908. *Enzyme and Microbial Technology*, 38(6): 848-854.

Carrillo-Pedroza, F. R., Nava-Alonso, F. and Uribe-Salas, A. 2000. Cyanide oxidation by ozone in cyanidation tailings: Reaction kinetics. *Minerals Engineering*, 13(5): 541-548.

Chapatwala, K. D., Babu, G. R. V., Vijaya, O. K., Kumar, K. P. and Wolfram, J. H. 1998. Biodegradation of cyanides, cyanates and thiocyanates to ammonia and carbon dioxide by immobilized cells of *Pseudomonas putida*. *Journal of Industrial Microbiology and Biotechnology*, 20(1): 28-33.

Chattopadhyay, S., Mukerji, A. and Sen, R. 2009. Biofuels. Biotechnology for agro-industrial residues utilisation, Springer**:** 61-76.

Chen, C. Y., Kao, C. M. and Chen, S. C. 2008. Application of Klebsiella oxytoca immobilized cells on the treatment of cyanide wastewater. *Chemosphere*, 71(1): 133-139.

Cluness, M. J., Turner, P. D., Clements, E., Brown, D. T. and O'Reilly, C. 1993. Purification and properties of cyanide hydratase from *Fusarium lateritium* and analysis of the corresponding *chy*1 gene. *Journal of general microbiology*, 139(8): 1807-1815.

Coello, M. D., Rodríguez-Barroso, M., Aragón, C. and Quiroga, J. 2010. Use of microbial activity measurements for monitoring RBC biofilms. *Environmental monitoring and assessment*, 169(1-4): 451-455.

Dakiky, M., Khamis, M., Manassra, A. and Mer'eb, M. 2002. Selective adsorption of chromium(VI) in industrial wastewater using low-cost abundantly available adsorbents. *Advances in Environmental Research*, 6(4): 533-540.

Dash, R. R., Balomajumder, C. and Kumar, A. 2009a. Removal of cyanide from water and wastewater using granular activated carbon. *Chemical Engineering Journal*, 146(3): 408-413.

Dash, R. R., Gaur, A. and Balomajumder, C. 2009b. Cyanide in industrial wastewaters and its removal: A review on biotreatment. *Journal of Hazardous Materials*, 163(1): 1-11.

Devuyst, E. A., Conard, B. R., Vergunst, R. and Tandi, B. 1989. A cyanide removal process using sulfur dioxide and air. *Journal of Minerals, Metals and Materials*, 41(12): 43-45.

Dogaris, I., Karapati, S., Mamma, D., Kalogeris, E. and Kekos, D. 2009. Hydrothermal processing and enzymatic hydrolysis of sorghum bagasse for fermentable carbohydrates production. *Bioresource technology*, 100(24): 6543-6549.

Donato, D. B., Nichols, O., Possingham, H., Moore, M., Ricci, P. F. and Noller, B. N. 2007. A critical review of the effects of gold cyanide-bearing tailings solutions on wildlife. *Environment international*, 33(7): 974-984.

Dubey, S. K. and Holmes, D. S. 1995. Biological cyanide destruction mediated by microorganisms. *World Journal of Microbiology and Biotechnology*, 11(3): 257-265.

Dumestre, A., Chone, T., Portal, J., Gerard, M. and Berthelin, J. 1997. Cyanide degradation under alkaline conditions by a strain of *Fusarium solani* isolated from contaminated soils. *Applied and Environmental Microbiology*, 63(7): 2729-2734.

Dursun, A. Y. and Aksu, Z. 2000. Biodegradation kinetics of ferrous(II) cyanide complex ions by immobilized *Pseudomonas fluorescens* in a packed bed column reactor. *Process Biochemistry*, 35(6): 615-622.

Dursun, A. Y., Çalık, A. and Aksu, Z. 1999. Degradation of ferrous(II) cyanide complex ions by *Pseudomonas fluorescens*. *Process Biochemistry*, 34(9): 901-908.

Eccles, H. 1995. Removal of heavy metals from effluent streams — Why select a biological process? *International Biodeterioration & Biodegradation*, 35(1–3): 5-16.

Edelstein, D. L. (2014). Mineral Commodity Summaries, U.S. Geological Survey**:** 196.

Enayathali, S. S. and Kumar, V. N. 2012. Performance of rotating biological contactor for treating segregated grey water for reuse. International Journal of Engineering Research and Technology, ESRSA Publications.

Ezzi, M. I. and Lynch, J. M. 2005. Biodegradation of cyanide by *Trichoderma sp.* and *Fusarium sp. Enzyme and Microbial Technology*, 36(7): 849-854.

Figueira, M. M., Ciminelli, V. S. T., Andrade, M. C. d. and Linardi, V. R. 1996. Cyanide degradation by an *Escherichia coli* strain. *Canadian Journal of Microbiology*, 42(5): 519-523.

Fry, W. E. and Millar, R. L. 1972. Cyanide degradion by an enzyme from *Stemphylium loti*. *Archives of Biochemistry and Biophysics*, 151(2): 468-474.

Guo, R., Chakrabarti, C. L., Subramanian, K. S., Ma, X., Lu, Y., Cheng, J. and Pickering, W. F. 1993. Sorption of low levels of cyanide by granular activated carbon. *Water Environment Research*, 65(5): 640-644.

Gupta, N., Balomajumder, C. and Agarwal, V. 2010. Enzymatic mechanism and biochemistry for cyanide degradation: a review. *Journal of Hazardous Materials*, 176(1): 1-13.

Gurbuz, F., Ciftci, H. and Akcil, A. 2009. Biodegradation of cyanide containing effluents by Scenedesmus obliquus. *Journal of Hazardous Materials*, 162(1): 74-79.

Gustavsson, J., Cederberg, C., Sonesson, U., Van Otterdijk, R. and Meybeck, A. 2011. Global food losses and food waste. *Food and Agriculture Organization of the United Nations, Rom*.

Hernández-Alonso, M. a. D., Coronado, J. M., Javier Maira, A., Soria, J., Loddo, V. and Augugliaro, V. 2002. Ozone enhanced activity of aqueous titanium dioxide suspensions for photocatalytic oxidation of free cyanide ions. *Applied Catalysis B: Environmental*, 39(3): 257-267.

Huang, C. P. and Wu, M. H. 1977. The removal of chromium(VI) from dilute aqueous solution by activated carbon. *Water Research*, 11(8): 673-679.

Hubbe, M. A., Hasan, S. H. and Ducoste, J. J. 2011. Cellulosic substrates for removal of pollutants from aqueous systems: A review. 1. Metals. *BioResources*, 6(2): 2161-2287.

Igwe, J. and Abia, A. 2007. Studies on the effects of temperature and particle size on bioremediation of AS (III) from aqueous solution using modified and unmodified coconut fiber. *Global Journal of Environmental Research*, 1(1): 22-26.

73

Cape Peninsula
University of Technology

Illán-Cabeza, N. A., Hueso-Ureña, F., Moreno-Carretero, M. N., Martínez-Martos, J. M. and Ramírez-Expósito, M. J. 2008. Synthesis, characterization and antiproliferative activity of metal complexes with the Schiff base derived from the condensation 1:2 of 2,6-diformyl-4-methylphenol and 5,6-diamino-1,3-dimethyluracil. *Journal of Inorganic Biochemistry*, 102(4): 647-655.

Kao, C. M., Liu, J. K., Lou, H. R., Lin, C. S. and Chen, S. C. 2003. Biotransformation of cyanide to methane and ammonia by Klebsiella oxytoca. *Chemosphere*, 50(8): 1055-1061.

Khodadadi, A., Abdolahi, M. and Teimoury, P. 2005. Detoxification of cyanide in gold processing wasterwater by hydrogen peroxide. *Iranian Journal of Environmental Health Science Engineering*, 2(3): 177-182.

Kim, S.-J., Lim, K.-H., Joo, K.-H., Lee, M.-J., Kil, S.-G. and Cho, S.-Y. 2002. Removal of heavy metal-cyanide complexes by ion exchange. *Korean Journal of Chemical Engineering*, 19(6): 1078-1084.

Klare, M., Scheen, J., Vogelsang, K., Jacobs, H. and Broekaert, J. A. C. 2000. Degradation of short-chain alkyl- and alkanolamines by $TiO_2$- and $Pt/TiO_2$-assisted photocatalysis. *Chemosphere*, 41(3): 353-362.

Kobayashi, M. and Shimizu, S. 2000. Nitrile hydrolases. *Current Opinion in Chemical Biology*, 4(1): 95-102.

Kowalska, M., Bodzek, M. and Bohdziewicz, J. 1998. Biodegradation of phenols and cyanides using membranes with immobilized microorganisms. *Process Biochemistry*, 33(2): 189-197.

Kwon, H. K., Woo, S. H. and Park, J. M. 2002. Degradation of tetracyanonickelate (II) by *Cryptococcus humicolus* MCN2. *FEMS Microbiology Letters*, 214(2): 211-216.

Leslie, J. F. and Summerell, B. A. (2006). The Fusarium laboratory manual, Blackwell publishing.

Li, J., Henriksson, G. and Gellerstedt, G. 2007. Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion. *Bioresource technology*, 98(16): 3061-3068.

Luque-Almagro, V., Blasco, R., Huertas, M., Martínez-Luque, M., Moreno-Vivián, C., Castillo, F. and Roldán, M. 2005. Alkaline cyanide biodegradation by *Pseudomonas pseudoalcaligenes* CECT5344. *Biochemical Society Transactions*, 33(Pt 1): 168-169.

Ma, J. and Dasgupta, P. K. 2010. Recent developments in cyanide detection: A review. *Analytica Chimica Acta*, 673(2): 117-125.

Maniyam, M. N., Sjahrir, F. and Ibrahim, A. L. 2011. Bioremediation of cyanide by optimized resting cells of Rhodococcus strains isolated from Peninsular Malaysia. *International Journal of Bioscience Biochemistry and Bioinformatics*, 1: 98-102.

Martínková, L., Vejvoda, V., Kaplan, O., Kubáč, D., Malandra, A., Cantarella, M., Bezouška, K. and Křen, V. 2009. Fungal nitrilases as biocatalysts: Recent developments. *Biotechnology Advances*, 27(6): 661-670.

74

Cape Peninsula
University of Technology

Mekuto, L., Jackson, V. A. and Ntwampe, S. K. O. 2013. Biodegradation of free cyanide using *Bacillus* sp. consortium dominated by *Bacillus safensis*, *Lichenformis* and *Tequilensis* strains: A bioprocess supported solely with whey. *Bioremediation and Biodegradation*, s18(004): 1-7.

Mitchell, D. A., Berovič, M. and Krieger, N. 2006. Solid-state fermentation bioreactor fundamentals: introduction and overview, Springer.

Monier, V. 2011. Preparatory study on food waste across EU 27, European Commission.

Montgomery, D. C. 2008. Design and analysis of experiments, John Wiley & Sons.

Mtui, G. Y. 2009. Recent advances in pretreatment of lignocellulosic wastes and production of value added products. *African Journal of Biotechnology*, 8(8).

Mulligan, C. N. 2005. Environmental applications for biosurfactants. *Environmental Pollution*, 133(2): 183-198.

Murthy, S., Bali, G. and Sarangi, S. 2012. Biosorption of lead by *Bacillus cereus* isolated from industrial effluents. *British Biotechnology Journal*, 2(2): 73-84.

Mussatto, S. I., Ballesteros, L. F., Martins, S. and Teixeira, J. A. 2012. Use of Agro-industrial wastes in solid-state fermentation processes. *Industrial waste. Croatia: InTech*: 121-40.

Myers, R. H., Montgomery, D. C. and Anderson-Cook, C. M. 2009. Response surface methodology: process and product optimization using designed experiments, John Wiley & Sons.

Nazly, N., Knowles, C. J., Beardsmore, A. J., Naylor, W. T. and Corcoran, E. G. 1983. Detoxification of cyanide by immobilised fungi. *Journal of Chemical Technology and Biotechnology*, 33(2): 119-126.

Nolan, L. M., Harnedy, P. A., Turner, P., Hearne, A. B. and O'Reilly, C. 2003. The cyanide hydratase enzyme of *Fusarium lateritium* also has nitrilase activity. *FEMS Microbiology Letters*, 221(2): 161-165.

Nsimba, E. B. (2009). Cyanide and cyanide complexes in the goldmine polluted land in the east and central rand goldfields. Unpublished MSc thesis, University of Witwatersrand, Johannesburg, South Africa.

Ntwampe, S. K. and Santos, B. A. 2013. Potential of Agro-Waste Extracts as Supplements for the Continuous Bioremediation of Free Cyanide Contaminated Wastewater. *International Journal of Agricultural, Biosystems Science and Engineering*, 7(7): 285-289.

O'Reilly, C. and Turner, P. D. 2003. The nitrilase family of CN hydrolysing enzymes – a comparative study. *Journal of Applied Microbiology*, 95(6): 1161-1174.

Oelofse, S. H. and Nahman, A. 2013. Estimating the magnitude of food waste generated in South Africa. *Waste Management and Research*, 31(1): 80-86.

Parolini, D. N. and Carcano, S. (2010). A model for cell growth in batch bioreactors.

Cape Peninsula
University of Technology

Patil, Y. and Paknikar, K. 2000. Development of a process for biodetoxification of metal cyanides from waste waters. *Process Biochemistry*, 35(10): 1139-1151.

Pereira, P. T., Arrabaça, J. D. and Amaral-Collaço, M. T. 1996. Isolation, selection and characterization of a cyanide-degrading fungus from an industrial effluent. *International Biodeterioration and Biodegradation*, 37(1–2): 45-52.

Potivichayanon, S. and Kitleartpornpairoat, R. 2014. Biodegradation of cyanide by a novel cyanide-degrading bacterium. *Biodegradation*, 1: 1362-1365.

Rao, M., Scelza, R., Scotti, R. and Gianfreda, L. 2010. Role of enzymes in the remediation of polluted environments. *Journal of Soil Science and Plant Nutrition*, 10(3): 333-353.

Raybuck, S. 1992. Microbes and microbial enzymes for cyanide degradation. *Biodegradation*, 3(1): 3-18.

Rhonas, O. C., William, T., Cameron, A., Andrew, L., Ross, S., Matthew, P., Saida, L., Johann, W., Ling, W., Erica, R., Samson, L., Sudheesh, N., Janette, T., Ryan, C., Sara, Z., Natalie, S.-G., Dante, A., Gregory, R. and Beverley, S. (2014). Gold Survey, Thomson Reuters**:** 120.

Rico, M., Benito, G., Salgueiro, A., Diez-Herrero, A. and Pereira, H. 2008. Reported tailings dam failures: a review of the European incidents in the worldwide context. *Journal of Hazardous Materials*, 152(2): 846-852.

Santos, B. A. Q. 2013. Continuous bioremediation of electroplating effluent. Chemical Engineering. Unpublished MTech thesis, Cape Peninsula University of Technology, Cape Town, South Africa**:** 98.

Santos, B. A. Q., Ntwampe, S. K. O. and Doughari, J. H. 2013a. Continuous biotechnological treatment of cyanide contaminated waters by using a cyanide resistant species of *Aspergillus awamori*. *Environmental Biotechnology–New Approaches and Prospective Applications, M. Petre, Ed. Croatia: InTech*: 123-146.

Santos, B. A. Q., Ntwampe, S. K. O., Doughari, J. H. and Muchatibaya, G. 2013b. Application of *Citrus sinensis* Solid Waste as a Pseudo-Catalyst for Free Cyanide Conversion under Alkaline Conditions. *BioResources*, 8(3): 3461-3467.

Sarla, M., Pandit, M., Tyagi, D. and Kapoor, J. 2004. Oxidation of cyanide in aqueous solution by chemical and photochemical process. *Journal of Hazardous Materials*, 116(1): 49-56.

Shukla, S. and Pai, R. S. 2005. Adsorption of Cu (II), Ni (II) and Zn (II) on modified jute fibres. *Bioresource Technology*, 96(13): 1430-1438.

Siedow, J. N. and Umbach, A. L. 1995. Plant mitochondrial electron transfer and molecular biology. *The Plant Cell*, 7(7): 821.

Silva-Avalos, J., Richmond, M. G., Nagappan, O. and Kunz, D. A. 1990. Degradation of the metal-cyano complex tetracyanonickelate (II) by cyanide-utilizing bacterial isolates. *Applied and Environmental Microbiology*, 56(12): 3664-3670.

Skowronski, B. and Strobel, G. A. 1969. Cyanide resistance and cyanide utilization by a strain of *Bacillus pumilus. Canadian Journal of Microbiology*, 15(1): 93-98.

Suh, Y.-J., Park, J. M. and Yang, J.-W. 1994. Biodegradation of cyanide compounds by *Pseudomonas fluorescens* immobilized on zeolite. *Enzyme and Microbial Technology*, 16(6): 529-533.

Wagner, A. M. and Krab, K. 1995. The alternative respiration pathway in plants: Role and regulation. *Physiologia Plantarum*, 95(2): 318-325.

Wang, J. and Chen, C. 2009. Biosorbents for heavy metals removal and their future. *Biotechnology Advances*, 27(2): 195-226.

Watanabe, A., Yano, K., Ikebukuro, K. and Karube, I. 1998. Cyanide hydrolysis in a cyanide-degrading bacterium, *Pseudomonas stutzeri* AK61, by cyanidase. *Microbiology*, 144(6): 1677-1682.

White, D. M. and Schnabel, W. 1998. Treatment of cyanide waste in a sequencing batch biofilm reactor. *Water Research*, 32(1): 254-257.

Wootton-Beard, P. C. and Ryan, L. 2011. A beetroot juice shot is a significant and convenient source of bioaccessible antioxidants. *Journal of Functional Foods*, 3(4): 329-334.

Young, C. and Jordan, T. 1995. Cyanide remediation: current and past technologies. Proceedings of the 10th annual conference on hazardous waste research, Kansas State University: Manhattan, KS.

Yu, B., Zhang, Y., Shukla, A., Shukla, S. S. and Dorris, K. L. 2000. The removal of heavy metal from aqueous solutions by sawdust adsorption — removal of copper. *Journal of Hazardous Materials*, 80(1–3): 33-42.

Zagury, G. J., Oudjehani, K. and Deschênes, L. 2004. Characterization and availability of cyanide in solid mine tailings from gold extraction plants. *Science of the Total Environment*, 320(2): 211-224.

Zilouei, H., Soares, A., Murto, M., Guieysse, B. and Mattiasson, B. 2006. Influence of temperature on process efficiency and microbial community response during the biological removal of chlorophenols in a packed-bed bioreactor. *Applied Microbiology and Biotechnology*, 72(3): 591-599.

Zou, S., Yao, S. and Ni, J. 2014. High-efficient nitrogen removal by coupling enriched autotrophic-nitrification and aerobic-denitrification consortiums at cold temperature. *Bioresource Technology*, 161: 288-296.

Cape Peninsula
University of Technology