



# **BIODEGRADATION OF CYANIDE AND SUBSEQUENT NITRIFICATION-AEROBIC DENITRIFICATION IN CYANIDE CONTAINING WASTEWATER**

by

**LUKHANYO MEKUTO**

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**Supervisor: Dr S.K.O. Ntwampe**

**Co-supervisor: Dr V.A. Jackson**

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## DECLARATION

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## ABSTRACT

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Environmental legislation focusing on wastewater disposal in industries that utilise cyanide and/or cyanide-related compounds has become increasingly stringent worldwide, with many companies that utilise cyanide products required to abide by the Cyanide International Code associated with the approval of process certifications and management of industries which utilise cyanide. This code enforces the treatment or recycling of cyanide-contaminated wastewater. Industries such as those involved in mineral processing, photo finishing, metal plating, coal processing, synthetic fibre production, and extraction of precious metals, that is, gold and silver, contribute significantly to cyanide contamination in the environment through wastewater. As fresh water reserves throughout the world are low, cyanide contamination in water reserves threatens not only the economy, but also endangers the lives of living organisms that feed from these sources, including humans. In the mining industry, dilute cyanide solutions are utilised for the recovery of base (e.g. Cu, Zn, Ni, etc.) and precious metals (e.g. Au, Ag, etc.). However, for technical reasons, the water utilised for these processes cannot be recycled upstream of the mineral bioleaching circuit as the microorganisms employed in mineral bioleaching are sensitive to cyanide and its complexes, and thus the presence of such compounds would inhibit microbial activity, resulting in poor mineral oxidation. The inability to recycle the water has negative implications for water conservation and re-use, especially in arid regions. A number of treatment methods have been developed to remediate cyanide containing wastewaters. However, these chemical and physical methods are capital intensive and produce excess sludge which requires additional treatment. Furthermore, the by-products that are produced through these methods are hazardous. Therefore, there is a need for the development of alternative methods that are robust and economically viable for the bioremediation of cyanide-contaminated wastewater.

Biological treatment of free cyanide in industrial wastewaters has been proved a viable and robust method for treatment of wastewaters containing cyanide. Several bacterial species, including *Bacillus sp.*, can degrade cyanide to less toxic products, as these microorganisms are able to use the cyanide as a nitrogen source, producing ammonia and carbon dioxide. These bacterial species secrete enzymes that catalyse the degradation of cyanide into several end-products. The end-products of biodegradation can then be utilised by the microorganisms as nutrient sources.

This study focused on the isolation and identification of bacterial species in wastewater containing elevated concentrations of cyanide, and the assessment of the cyanide biodegradation ability of the isolates. Thirteen bacterial isolates were isolated from

electroplating wastewater by suppressing the growth of fungal organisms and these species were identified as species belonging to the *Bacillus* genus using the 16S rDNA gene. A mixed culture of the isolates was cultured in nutrient broth for 48 hours at 37°C, to which F-CN as KCN was added to evaluate the species' ability to tolerate and biodegrade cyanide in batch bioreactors. Subsequently, cultures were supplemented solely with agro-waste extracts, that is, *Ananas comosus* extract (1% v/v), *Beta vulgaris* extract (1% v/v), *Ipomea batatas* extract (1% v/v), spent brewer's yeast (1% v/v) and whey (0.5% w/v), as the primary carbon sources. Owing to the formation of high ammonium concentration from the cyanide biodegradation process, the nitrification and aerobic denitrification ability of the isolates, classified as cyanide-degrading bacteria (CDB) was evaluated in a batch and pneumatic bioreactor in comparison with ammonia-oxidising bacteria (AOB). Furthermore, the effects of F-CN on the nitrification and aerobic denitrification was evaluated assess the impact of F-CN presence on nitrification. Additionally, optimisation of culture conditions with reference to temperature, pH and substrate concentration was evaluated using response surface methodology. Using the optimised data, a continuous biodegradation process was carried out in a dual-stage packed- bed reactor combined with a pneumatic bioreactor for the biodegradation of F-CN and subsequent nitrification and aerobic denitrification of the formed ammonium and nitrates.

The isolated bacterial species were found to be gram positive and were able to produce endospores that were centrally located; using the 16S rDNA gene, the species were found to belong to the *Bacillus* genus. The species were able to degrade high cyanide concentration in nutrient broth with degradation efficiencies of 87.6%, 65.4%, 57.0% and 43.6% from 100 mg F-CN/L, 200 mg F-CN/L, 300 mg F-CN/L, 400 mg F-CN/L and 500 mg F-CN/L respectively over a period of 8 days. Additionally, the isolates were able to degrade cyanide in an agro-waste supported medium, especially in a medium that was supplemented with whey which achieved a degradation efficiency of 90% and 60% from 200 mg F-CN/L and 400 mg F-CN/L, respectively over a period of 5 days. The nitrification ability of the isolates was evaluated and the removal of  $\text{NH}_4^+/\text{NO}_3^-$  by the CDB and AOB in both shake flasks and pneumatic bioreactor was determined to be pH dependent. The maximum  $\text{NH}_4^+/\text{NO}_3^-$  removal evaluated over a period of 8 days for CDB and 15 days for AOB, observed at pH 7.7 in shake flasks, was 75% and 88%, respectively, in the absence of F-CN. Similarly, the removal of  $\text{NH}_4^+/\text{NO}_3^-$  in a pneumatic bioreactor was found to be 97.31% for CDB and 92% for AOB, thus demonstrating the importance of aeration in the designed process. The nitrification by CDB was not inhibited by cyanide loading up to a concentration of 8 mg F-CN/L, while the AOB were inhibited at cyanide loading concentration of 1 mg F-CN/L. The CDB removed the  $\text{NH}_4^+/\text{NO}_3^-$  in PBSs operated in a fed-batch mode, obtaining efficiencies >99% ( $\text{NH}_4^+$ ) and 76 to 98% ( $\text{NO}_3^-$ ) in repeated cycles (n = 3) under F-CN ( $\leq 8$  mg F-CN/L).

The input variables, that is, pH, temperature and whey-waste concentration, were optimised using a numerical optimisation technique where the optimum conditions were found to be: pH 9.88, temperature 33.60 °C and whey-waste concentration 14.27 g/L, under which 206.53 mg CN/L in 96 h can be biodegraded by the microbial species from an initial cyanide concentration of 500 mg F-CN/L. Furthermore, using the optimised data, cyanide biodegradation in a continuous mode was evaluated in a dual-stage packed-bed bioreactor connected in series to a pneumatic bioreactor system used for simultaneous nitrification including aerobic denitrification. The whey-supported *Bacillus sp.* culture was not inhibited by the free cyanide concentration of up to 500 mg F-CN/L, with an overall degradation efficiency of  $\geq 99\%$  with subsequent nitrification and aerobic denitrification of the formed ammonium and nitrates over a period of 80 days.

## DEDICATION

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**“Art is science; it is the science of freedom!”** Cindy-Jade Africa

This quote provoked a profound realization, that science is in fact an art!  
More specifically (to me) it is the art of will, where will, in addition to one having strong resolve, encompasses the capability of conscious choice, decision and intention.  
Art and science co-exist synergistically, creating a wealth and coalescence of freedom.  
Freedom of imagination, mental flexibility and fortitude!

Both (science and art) can be groomed,  
Both can be beautiful,  
And both can be painfully obscure!

I dedicate this work to all who are willing to take up the challenge and embrace the opportunity and their ability to be “free”. I dedicate this to; those who taught me, those who instilled in me the importance of curiosity and a want for knowledge, those who nurtured and encouraged me and my enthusiasm for science, those whose faith in me made me believe in myself and my capability, those who inspired me to be and do more. For your help, encouragement and inspiration I will be forever grateful and I thank you.

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## RESEARCH OUTPUTS

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The following research outputs represent contributions by the candidate to scientific knowledge and development during his master's candidacy (2013-2014):

### ➤ Publications

Mekuto, L., Ntwampe, S.K.O. & Jackson, V.A. Biodegradation of free cyanide using *Bacillus safensis*, *Lichenformis* and *Tequilensis* strains: A bioprocess supported solely with whey. *Journal of Bioremediation and Biodegradation*, in Press, S:18

Mekuto, L., Ntwampe, S.K.O. & Jackson. Nitrification and aerobic denitrification of high strength ammonia wastewater containing free cyanide using a *Bacillus* consortium. submitted to *Environmental Science and Pollution Research*

Mekuto, L., Ntwampe, S.K.O. & Jackson. Optimisation of simultaneous free cyanide, ammonia and nitrate biodegradation in a continuous novel dual-stage packed-bed in series with a pneumatic bioreactor system. Submitted to *Biodegradation*

### ➤ Presentations

**Mekuto, L., Ntwampe, S.K.O. & Jackson, V.A.**, 4<sup>th</sup> Municipal Water Quality conference, 8 – 11 July 2013, Sun City, South Africa, Paper: Biodegradation of free cyanide using bacterial species isolated from electroplated wastewater.

**Mekuto, L, Ntwampe, S.K.O. & Jackson V.A.**, 4<sup>th</sup> World congress on Biotechnology, 23- 25 September 2013, Raleigh, North Carolina, USA, Paper: Biodegradation of free cyanide using *Bacillus safensis*, *Lichenformis* and *Tequilensis* strains: A bioprocess supported solely with whey



## LAYOUT OF THESIS

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The overall aim of the study was to isolate cyanide degrading bacteria that and utilise these organisms for the biodegradation and/or removal of free cyanide, ammonium and nitrates in batch and continuous system. The references are listed at the end as a separate chapter in accordance with the Harvard method of referencing.

The thesis is subdivided into the following chapters:

- **Chapter 1**, the introduction, provides the background information on cyanide. It also presents the problem statement, hypothesis, research objectives, the significance of the study and the delineation of the study.
- **Chapter 2**, the literature review, provides formation on the chemistry and toxicity of cyanide to the environment and humans. It also entails information of current remedial processes that are used to treat cyanide containing environments and their advantages and disadvantages. The review entails information on the use of organic waste materials for supplementation and removal of contaminants in the environment.
- **Chapter 3**, the research methodology. It summaries the materials and methods used in this study to determine cyanide. Information on culture preparation, contaminant determination and bioreactor operation are also discussed.
- **Chapter 4** is the results and discussion chapter.
- **Chapter 5**, overall conclusion and recommendations chapters, presents the answers to the research questions in Chapter 1 while also listing recommendations for future research.

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## LIST OF SYMBOLS

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### Nomenclature

<u>Symbol</u>	<u>Description</u>	<u>Units</u>
$\beta_0$	Constant	-
$\beta_i$	Linear coefficient	-
$\beta_{ii}$	Quadratic coefficient	-
$\beta_{ij}$	Interactive coefficient	-
$CN_B^-$	Biodegraded cyanide	mg/L
$CN_R^-$	Residual cyanide	mg/L
$CN_S^-$	Initial cyanide	mg/L
$CN_V^-$	Volatilized cyanide	mg/L
$CN_{Vf}^-$	Final cyanide in control	mg/L
$CN_{Vo}^-$	Initial cyanide in control	mg/L
$R^2$	Goodness of model fit	-
$X_i$	Coded independent variables	Units not defined
$Y$	Response variable	Units not defined

### Greek symbols

<u>Symbol</u>	<u>Description</u>	<u>Units</u>
$\varepsilon$	Error	Units not defined

### Subscripts

<u>Symbol</u>	<u>Description</u>	<u>Units</u>
A	pH	-
B	Substrate concentration	g/L
C	Temperature	° C



## GLOSSARY

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Abbreviation	Description
<i>A. comosus</i>	<i>Ananas comosus</i>
AOB	Ammonia oxidising bacteria
BC	Batch culture
<i>B. vulgaris</i>	<i>Beta vulgaris</i>
CCD	Central composite design
CDB	Cyanide degrading bacteria
CN <sub>SAD</sub>	Strong acid dissociable cyanide
CN <sub>WAD</sub>	Weak acid dissociable cyanide
DNS	Dinitrosalicylic acid
F-CN	Free cyanide
HCN	Hydrogen cyanide
<i>I. batatas</i>	<i>Ipomoea batatas</i>
KCN	Potassium cyanide
NH <sub>4</sub> <sup>+</sup> -N	Ammonium nitrogen
NO <sub>3</sub> <sup>-</sup> -N	Nitrate nitrogen
PBB	Packed bed bioreactor
PBS	Pneumatic bioreactor system
PCR	Polymerase chain reaction
RSM	Response surface methodology
SBY	Spent brewer's yeast
SCN	Thiocyanate

### Clarification of Terms

Cyanide: is a carbon nitrogen chemical compound with the formula  $-C\equiv N$ .

Bioremediation: cleaning of polluted water systems with microorganisms.

Biofilm: Clusters of microbial cells normally attached to a surface.

Inoculum: Microbial culture prepared for addition into a solution to seed the reactor with microbial activity

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# **CHAPTER 1**

# **INTRODUCTION**

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# CHAPTER 1

## INTRODUCTION

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### 1.1 Background

Cyanide is a natural compound that is produced by living organisms such as bacteria, algae fungi and plants as a defensive mechanism to limit predation, and in some cases as an invasive mechanism (Luque-Almagro *et al.*, 2005). It is also found in the stratosphere and non-urban troposphere as a result of natural sources, including the burning of biomass and gasses from volcanoes. These natural sources of cyanide in the environment are however insignificant compared with anthropogenic sources. The mineral processing, electroplating and plastics industries contribute significantly to the presence of cyanide in the environment (Bosecker & Blumenroth, 2001, Branch, 1994). An estimated 60 000 tons of sodium cyanide were produced in 2001 and the mineral processing industry utilised 90% of this compound for gold extraction (Van Zyl *et al.*, 2011).

Free cyanide (F-CN) and metal-complexed cyanide contaminants are mostly produced by the mineral-processing and electroplating industries. The occurrence of high concentrations of thiocyanate and metal-complexed cyanides from the mineral-processing industry is mainly due to the interaction of free cyanide with sulphur and metal species in the ore during the cyanidation process (Gould *et al.*, 2012). Free cyanide is the most toxic form of cyanide but it has a short half-life, owing to its volatile nature. However, the metal-complexed cyanides and thiocyanates can accumulate in the environment owing to their high stability, especially in an alkaline environment.

The dissociation of metal-complexed cyanides in acidic solutions and/or soils causes environmental contamination and thus deterioration as the dissociation of such complexes ultimately results in the formation of free cyanide. Cyanide in soil has been found to form complexes with metallic species, thus forming stable cyanide compounds that are less toxic. This results in the prolonged presence of the cyanides, and thus their gradual decomposition by microbial species for extended periods, ensuring the gradual release of free cyanide into the soil. Although microbial flora contributes significantly to the degradation of cyanide compounds in soil (Taylor *et al.*, 2006), there is limited information about the gradual release of free cyanide from its complexes as a result of microbial degradation in contaminated soil. Furthermore, microbial species have a low tolerance to F-CN, with the maximum of 200 mg F-CN/L being the highest concentration which most cyanide-degrading microorganisms can tolerate (Kuyucak & Akcil, 2013); resulting in an extended period of ecological recovery for heavily contaminated soil.

One of many ways in which cyanide is discharged into the environment is through wastewater discharge. The cyanide into surface waters does not only pose a threat to aquatic organisms but also to organisms that utilise surface water terrestrially. The most common in which cyanide-contaminated water is discharged is through municipal systems to wastewater treatment plants. The organisms normally employed for the treatment of such wastewater become susceptible to cyanide, reducing the efficacy of the systems (Kim *et al.*, 2011a; Kim *et al.*, 2011b; Kim *et al.*, 2011c). In most instances, the treated water from the municipal treatment plants is ultimately discharged into rivers which can lead to accumulation of cyanide (particularly in its metal-complex form) in river sediment, including riparian wetlands. This leads to contamination of water tables downstream in the region, such as borehole water sources, resulting in increasing health risks to animals and humans. In this case, the toxicity of cyanide lies in its ability to form complexes with a number of transition metals that are mainly associated with functional proteins, that is, iron in cytochrome oxidase.

Chemical, physical and biological processes can be employed to remediate cyanide-contaminated wastewaters, including soils. The use of chemical and physical methods such as the sulphur dioxide process and the use of hydrogen peroxide are unfavourable due to high capital and operational costs and production of excess sludge containing other environmental contaminants. This excess sludge produced must then also be disposed off in the form of heaps, which poses an additional and significant risk to the environment as the resultant by-products in some of the treatment processes result in the formation of hazardous chemicals such as sulphuric acid (Akciil and Mudder, 2003; Mosher and Figueroa, 1996).

The application of microorganisms in the treatment of cyanide has been found to be the most robust and effective process for the removal of cyanide in wastewaters (Akciil & Mudder, 2003, Kao *et al.*, 2004, Karavaiko *et al.*, 2000), where microbial species convert cyanide to ammonium and other less toxic products. The ammonium produced is metabolised as a nitrogen source by the microorganisms. The degradation of cyanide by a particular organism is dependent on the degradation pathway. Current research focuses on the utilisation of renewable resources such as agricultural waste, through assisted biostimulation and bioaugmentation, for the biodegradation of cyanide (Ntwampe & Santos, 2013; Santos *et al.*, 2013c). Although biological systems have high capital costs, due to the addition of refined nutrients to sustain the microorganisms, operating costs remain low.

The use of agro-waste materials as a source of carbon and micro-nutrients for cyanide-degrading organisms lowers the capital costs while improving process performance.

This study focuses on the biological treatment methods for cyanide-contaminated waters, particularly from the mineral processing and electroplating industries, with the main emphasis on the biological degradation of cyanide. Furthermore, the study evaluates the application of agricultural waste materials as primary supplements to sustain the microbial communities utilised in such bioprocesses.

## **1.2 Problem statement**

The use of cyanide-based compounds in the mining industry for the extraction of precious metals poses a significant threat to the environment and surrounding communities, as it is a poisonous compound. It can thus contaminate water sources and/or react with other substances to form other cyanide-based compounds that could result in either health-related complications or even death. The toxicity of cyanide, coupled with its continuous use and discharge in mining operations, necessitates the development of environmentally friendly remediation processes to decontaminate the generated toxic waste. The processes that are currently employed for the remediation of cyanide-containing wastewaters result in end products that are hazardous to the environment. Furthermore, these processes are capital intensive and do not treat some of the cyanide complexes. Therefore, a biological process is proposed for the remediation of cyanide-containing wastewaters so that they could be re-used or meet regulatory discharge limits.

## **1.3 Hypothesis**

It is hypothesised that the microbial species isolated from the cyanide-containing wastewater are able to tolerate and biodegrade free cyanide and utilise the formed products (ammonium and nitrates), both in batch and continuous system. Furthermore, it is hypothesised that an increase in cyanide concentration will negatively affect the performance of the microbial community.

## **1.4 Research objectives**

The objectives of this study were to:

- isolate cyanide tolerant and degrading microorganisms from cyanide-contaminated wastewater;
- characterise and identify the isolated microorganisms using molecular techniques;
- investigate the potential application of agricultural residues as supplements for the microbial community for optimal cyanide biodegradation;
- investigate the potential of the cyanide-degrading microorganisms to utilise the ammonium and nitrates that formed through cyanide biodegradation;
- Optimise the physicochemical conditions for successful cyanide biodegradation;

- Construct a lab-scale biofilm reactor and determine the biodegradation of free cyanide in continuous mode at optimal conditions.

### **1.5 Significance of the study**

In this study, a robust and efficient bioprocess was developed for continuous degradation of cyanide containing wastewater. This study should assist the mining and mineral-processing industries in South Africa in the successful biodegradation of cyanide and its by-products generated through the cyanidation process, so that the water can be re-used upstream for various processes. This should save costs and the amount of water that is used since South Africa is a semi-arid country. Furthermore, the study also showed the application of organic waste products as supplements to promote the microbial communities in the bioprocess, thus lowering reagent and operating costs while promoting successful bioremediation of cyanide.

### **1.6 Delineation of the study**

The following factors were not considered in this study:

- Mathematical modelling of the biodegradation process.
- Biosorption of heavy metals.
- Cyanide degradation kinetics.
- Environmental impact of cyanide.
- Enzymology of cyanide degradation.

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# **CHAPTER 2**

# **LITERATURE REVIEW**

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## CHAPTER 2

### LITERATURE REVIEW

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#### 2.1 Cyanide: chemistry, types, environmental and anthropogenic sources, uses and production

##### 2.1.1 Cyanide Chemistry

Cyanides are chemical compounds which contain the cyano group (CN), consisting of a carbon atom triple-bonded to a nitrogen atom. The most toxic form of cyanide is the free cyanide ion (CN<sup>-</sup>) and hydrogen cyanide gas (HCN). Cyanide compounds are toxic, carcinogenic and mutagenic. Cyanide occurs in different forms in the environment; including gaseous, aqueous and solid forms. The toxicity and reactivity of these cyanide species is dependent on their form, which determines their environmental fate and transport (Nsimba, 2009). Hydrogen cyanide (HCN), cyanogen chloride (CNCl), and cyanogen bromide (CNBr) are the three gaseous forms of cyanide. All these gases are highly toxic to humans and animals if they are inhaled or dermally absorbed. These gases are highly soluble in water but can be easily hydrolysed to form cyanate which subsequently degrades to ammonia (NH<sub>3</sub>) and carbon dioxide (CO<sub>2</sub>) at alkaline pH conditions (Nsimba, 2009).

Aqueous cyanide in industrial effluents can be classified in five categories: (1) free cyanide, in the form of hydrogen cyanide (HCN) and cyanide ion (CN<sup>-</sup>); (2) simple cyanides, in the form of sodium cyanide (NaCN) and potassium cyanide (KCN); (3) thiocyanate (SCN); (4) weak acid dissociable cyanides (CN<sub>wad</sub>), which are mainly metal-complexed cyanides consisting of metal species such as Zinc (Zn), Copper (Baxter & Cummings), Nickel (Ni), amongst others; and (5) strong acid dissociable cyanides (CN<sub>sad</sub>), which are strong complexes with metal species such as Iron (Fe), Cobalt (Co), Gold (Au) and Silver (Ag). The toxicity of these cyanide compounds decreases from category one to five (Ebbs, 2004, Zagury *et al.*, 2004). The aqueous forms of cyanide are listed in Table 2.1.



**Table 2.1:** General categorisation of cyanides (Bhalla *et al.*, 2012)

Types of cyanide	Examples	Remarks
Free	HCN, CN <sup>-</sup>	Equilibrium depends on pH (pKa 9.24 at 25°C)
Simple	KCN, NaCN, Ca(CN) <sub>2</sub>	Ionise in aqueous solutions and are present as HCN below pH 8.
Complex	CN <sub>WAD</sub> and CN <sub>SAD</sub>	CN <sub>WAD</sub> ionise quickly and CN <sub>SAD</sub> are very stable and difficult to ionise
Inorganic	SCN <sup>-</sup> , CNO <sup>-</sup>	Cyanate unstable
Organic	Benzonitrile, acetonitrile, acrylonitrile	Stable

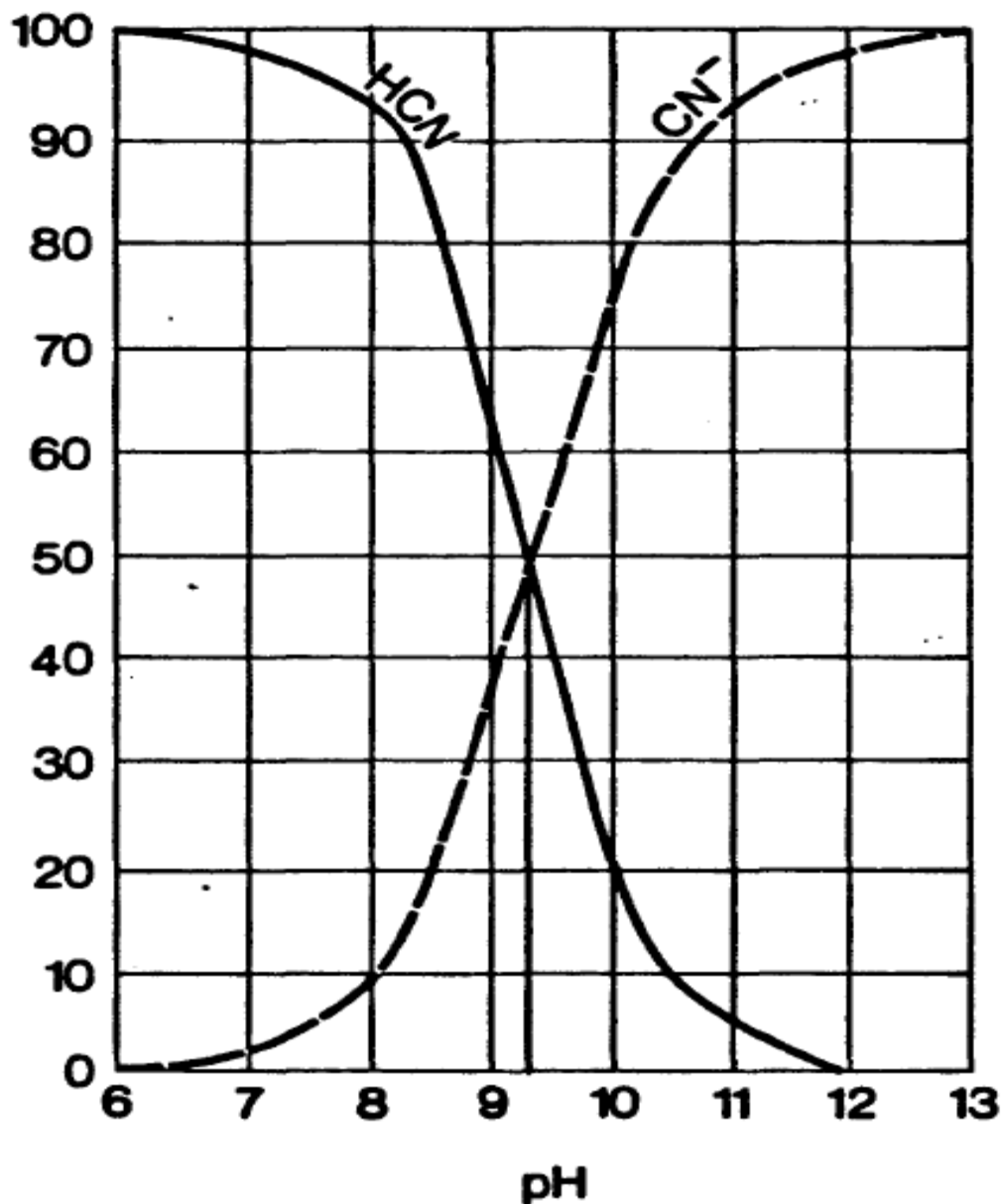
### 2.1.1.1 Free cyanide

Free cyanide (F-CN) exists in water in many forms, either as a cyanide ion (CN<sup>-</sup>) or as hydrogen cyanide. HCN is a weak acid with a pKa value of 9.24 at 25°C, and is soluble in water and alcohol. HCN is highly volatile and mostly escapes as HCN gas under appropriate conditions. Industrially, phosphoric acid is used to stabilise hydrogen cyanide because of its instability. The properties of HCN are listed in Table 2.2.

**Table 2.2:** Physical and chemical properties of HCN (WHO, 2004)

Property	Value
Relative molecular mass	27.3
Boiling point (°C)	25.70
Solubility (30°C)	Miscible with water Soluble in alcohol
Specific density: vapours (31°C)	0.937
Odour threshold	0.7 mg/m <sup>3</sup> in air 0.17 mg/L in water
Henry's law constant (dimensionless)	180 to 300
Octanol/water partition coefficient (log K <sub>ow</sub> )	0.66
Vapour pressure (kPa)	35.2 at 0°C 107.2 at 27.2°C

Physicochemical parameters such as pH and temperature play a role in the HCN/CN ratio in solution. By increasing or decreasing the pH of the solution at a constant temperature (25°C), a ten-fold decrease or an increase in the HCN/CN ratio occurs as shown in Figure 2.1. The ratio of HCN and CN are equal at a pH of 9.24 (Simovic, 1984). HCN has a relatively high vapour pressure (53.1 kPa at 10.2°C) and can easily volatilise into the atmosphere, even in stagnant solutions (Simovic, 1984). The equilibrium constant ( $K_a$ ) varies slightly with temperature. As illustrated in Figure 2.1, the stability of HCN is highly dependent on pH, with HCN easily dissociating into  $CN^-$  in highly alkaline solutions.



**Figure 2.1:** Relative percentages of HCN and  $CN^-$  in solution as a function of pH (Simovic, 1984)

Free cyanide is the most toxic form of cyanide and its presence in soils, surface and ground waters can result in serious damage to the environmental and geochemical cycles. When released into the environment, cyanide reacts with a number of species, thus providing numerous ways for its degradation and attenuation, such as adsorption, volatilisation, complexation, precipitation and biodegradation. Although F-CN reacts readily in the environment and forms complexes of varying stabilities, it is still toxic to living organisms at very low concentrations (Donato *et al.*, 2007, Logsdon *et al.*, 1999).

The presence of F-CN and complexes in the environment has been responsible for high mortality rates of wildlife (Donato *et al.*, 2007), as aquatic organisms are very sensitive to cyanide. The presence of F-CN has shown to reduce the swimming performance and reproduction of fish. Additionally, delayed mortalities, disrupted respiration and altered growth patterns are some of the effects that F-CN has on aquatic species (Logsdon *et al.*, 1999). Contamination of water reserves, that is, groundwater that serves as source of water for inland organisms, poses a huge safety concern. With most water reserves throughout the world running dry, such water reserves serve as alternative sources of water for a number of uses. Life-threatening situations as a result of F-CN contamination in groundwater can result, with the F-CN acting against core enzymes that are responsible for oxygen transfer, ultimately resulting in death (Donato *et al.*, 2007, Kjeldsen, 1999, Logsdon *et al.*, 1999).

#### 2.1.1.2 Simple cyanides

Simple cyanides are cyanides available in a salt form where the cyanide ion is bonded to alkali metals such as sodium (Na) and potassium (K). The cyanide salts can easily dissociate to their ionic components as shown in Equation 2.1.



Where M/M<sup>+</sup> is an alkali metal. The hydrolysis of alkali metal cyanides such as NaCN in solution results in the formation of molecular hydrogen cyanide and free hydroxyl groups (Equation 2.2):



The HCN formed is weakly dissociated and only forms when large quantities of hydrogen ions are available in solution; thus hydroxyl groups dominate, increasing the pH of the

solution to alkaline conditions. The decrease in pH thus favours the formation of HCN as previously shown in Figure 2.1 (Kuyucak & Akcil, 2013, Simovic, 1984).

### 2.1.1.3 Complexed cyanides

Cyanide is highly reactive and can easily form complexes with a variety of compounds. During the cyanidation process, cyanide forms complexes with metals and sulphur species from sulphur containing ore. Cyanide reacts with sulphur species to form thiocyanate ( $\text{SCN}^-$ ) (Equation 2.3). Thiocyanate is also produced naturally through the activity of enzymes such as sulphur-transferases which transfer sulphur species from thiosulphate to the cyanide ion, thus forming thiocyanate (Equation. 2.4) (Gould *et al.*, 2012, Gupta *et al.*, 2010).



Other cyanide complexes are formed with various metals such as Cu, Zn, Ni, Fe and other transition metals (Baxter & Cummings, 2006). These species are generally anionic and have a general formula of  $\text{M}(\text{CN})_x^{n-}$ , where M represent a metal ion. The stability of the complexes is dependent on the properties of the metal that is complexed to the cyanide; therefore metal-complexed cyanides are categorised into two groups; weak acid dissociable cyanides ( $\text{CN}_{\text{WAD}}$ ) and strong acid dissociable cyanides ( $\text{CN}_{\text{SAD}}$ ) (Akcil & Mudder, 2003)

$\text{CN}_{\text{WAD}}$  are metal-complexed cyanides that require mildly acidic conditions to dissociate ( $\text{pH} \leq 4$ ). These metal complexes include  $\text{Cu}(\text{CN})_3^{2-}$ ,  $\text{Ni}(\text{CN})_4^{2-}$ ,  $\text{Zn}(\text{CN})_4^{2-}$ ,  $\text{Cd}(\text{CN})_4^{2-}$  and  $\text{Hg}(\text{CN})_2$ .  $\text{CN}_{\text{SAD}}$  require acidic conditions to dissociate the metal complex ( $\text{pH} \leq 1$ ). These metal complexes include  $\text{Au}(\text{CN})_2^-$ ,  $\text{Ag}(\text{CN})_2^-$ ,  $\text{Fe}(\text{CN})_6^{4-}$ ,  $\text{Fe}(\text{CN})_6^{3-}$ ,  $\text{Pt}(\text{CN})_4^{2-}$  and  $\text{Co}(\text{CN})_6^{3-}$  (Nsimba, 2009).

Furthermore, the cyano group is capable of forming complexes with organic molecules to form organocyanide complexes. The organic residue is normally attached to the carbon atom of the cyanide via a covalent bond. Common examples include nitriles such as acetonitrile ( $\text{CH}_3\text{CN}$ ) and acrylonitrile which are industrially utilised for manufacturing nylon, plastics and pesticides (Bhalla *et al.*, 2012). Vitamin B12 is also an organocyanide compound which is consumed by humans as a source of vitamin (Nsimba, 2009).

### 2.1.2 Cyanide presence in the environment: sources

Natural and anthropogenic activities both contribute to the presence of cyanide in the environment. Industrial activities are the most significant sources of environmental cyanide contamination compared with natural processes. Some of the major sources of cyanide from natural and industrial sources are listed in Table 2.3.

**Table 2.3:** Natural and anthropogenic sources of cyanide

<b>Natural sources</b>	<b>Anthropogenic sources</b>
<b>Cyanogenic algae</b>	Electroplating industry
<i>Chlorella vulgaris</i>	Mineral processing
<i>Plectonema boryanum</i>	Processing of cyanogenic crops
<i>Anacystis nidulans</i>	Pharmaceutical industry
	Paint-manufacturing industry
<b>Cyanogenic bacteria</b>	Coal-coking plants
<i>Pseudomonas aeruginosa</i>	Coal-gasification plants
<i>Pseudomonas fluorescens</i>	Synthetic fibre-producing plants
<i>Chromobacterium violaceum</i>	Manufactured gas plants
	Photo-finishing industry
<b>Cyanogenic fungi</b>	Plastics industry
<i>Stemphylium loti</i>	Agricultural sector
<i>Marasmius oreades</i>	
<b>Cyanogenic crops</b>	
<i>Manihot esculenta</i> (cassava)	
<i>Malus domestica</i> (apple)	
<i>Phaseolus lunatus</i> (Lima beans)	

#### 2.1.2.1 Naturally occurring cyanides

Cyanide is produced naturally by bacteria, fungi, algae and plants. Several plants species are a significant source of cyanide. They produce cyanide as a defence mechanism against herbivores and pathogens. All these plants produce cyanide in small quantities and at low concentrations. A plant is considered cyanogenic when it produces 10 mg per kg of plant material. In most instances, the bitter taste in plants is a result of cyanide glycosides which are hydrolysed through enzymatic action to produce hydrogen cyanide. The following reaction occurs in cyanogenic plants when their tissue is damaged by herbivores (Nsimba, 2009). Another source of cyanide gas in the environment results from forest fires; owing to

the incomplete combustion of substances containing nylon, this produces cyanide through depolymerisation (Li *et al.*, 2000). The cyanogenic plants and trees produce cyanide gas during forest fires because of the thermal hydrolysis of the glycosides present with the plants and trees.

In nutrient-limited environments, a number of fungi and bacteria synthesise cyanide compounds to inhibit the growth of other microbial species competing for the same nutritional source. Bacteria such as *Chromobacterium violaceum* ATCC 53434 produce an isonitrile antibiotic that is active against Gram-positive bacteria, while *Trichoderma harzianum* produces homothalin II, a nitrile that is active against fungi, as well as Gram-positive and Gram-negative bacteria (Baxter & Cummings, 2006). Cyanide production in fungi is normally produced during the late exponential and/or early stationary phase of growth, in fruiting bodies and mycelia. For some species, amino acids such as glycine are used for the synthesis of cyanide where the carbon from the methylene group in glycine is donated for cyanide synthesis (Knowles, 1976).

Cyanogenesis in bacterial species such as *Pseudomonas aeruginosa* requires the presence of amino acids in the growth medium. It has been proposed that cyanogenesis in bacterial species is mainly for the control and limitation of high concentrations of intercellular glycine and for inhibiting the growth of competing species (Zlosnik *et al.*, 2006). Cyanide in bacterial species is produced as a secondary metabolite and thus its synthesis is mainly accomplished in nutrient-deprived environments or when there are limited quantities of dissolved oxygen for metabolic processes. Oxygen (O<sub>2</sub>), phosphorus (P) and iron (Fe) concentration play a vital role in cyanogenesis for most bacterial species, particularly under micro-aerophilic conditions. The cyanide formed through cyanogenesis can also form complexes with transition metal ions available in soil and this mechanism assists the organism in utilising the metal ions needed for growth. Cyanide concentrations up to 300µM can be produced by bacterial species such as *Pseudomonas aeruginosa* (Blumer & Haas, 2000, Cipollone *et al.*, 2007, Faramarzi & Brandl, 2006, Gallagher & Manoil, 2001, Zlosnik *et al.*, 2006). However, these sources of cyanide are minuscule when compared to anthropogenic sources, which largely contribute to a variety of cyanides in the environment.

#### **2.1.2.2 Anthropogenic sources of cyanide**

A major source of cyanide compounds throughout the world is through anthropogenic activities from various industrial sectors. Industries such as those that produce plastics, electroplated goods, organic chemicals, photographic films, pharmaceuticals, and precious metals, use a variety of cyanides as one of the primary agents facilitating the production of

high and/or added-value products. Furthermore, the coking of coal, including gasification, processing of ore to produce iron, steel, aluminium and petroleum products, also contributes to the presence of cyanide in various forms in the environment (Akcil, 2003, Nsimba, 2009). The extent to which individual countries contribute to environmental cyanide contamination is largely dependent on the level of industrialisation. In the USA, it is estimated that 18 billion litres of cyanide waste are generated annually (Baxter & Cummings, 2006), and in most instances, it is produced in the form of metal complexes which may cause ecosystem disturbances and general environmental deterioration (Nsimba, 2009).

Similarly, the application of nitrile pesticides such as bromoxynil and chlorothalonil in the agricultural sector also contributes to cyanide contamination in agricultural produce and cultivated land. Bromoxynil is used as a herbicide for the control of diseases of broad-leaf crops, while chlorothalonil is used as a fungicide and as an anti-fouling agent in boat hulls. The repeated application of these compounds combined with sustained irrigation and generated runoff from agricultural land during rainy seasons, may lead to cyanide contamination in the receiving surface waters. Furthermore, during the cultivation of cassava, significant concentrations of cyanogenic glycosides are produced. When these cyanogenic glycosides are hydrolysed through enzymatic action, free cyanide is released (Siller & Winter, 1998). However, there are limited studies that have focused and reported on the quantities of cyanide and/or cyanogenic glycosides released from various agricultural crops into agricultural soil. In general, this means that cyanide and/or cyanogenic glycosides loading into agricultural soil from agricultural produce is largely undocumented.

## **2.2 Degradation processes for cyanide containing wastes**

### **2.2.1 Natural, physical and chemical degradation of cyanide**

The continued use and generation of cyanide in industries that utilise cyanide have led to the development and subsequent implementation of processes that degrade cyanide to acceptable discharge levels. Factors that are taken into consideration when selecting an appropriate treatment method are based on the chemical characteristics of the waste, the volume of the waste that is to be treated, the environmental setting of the treatment site and the regulations that are in place to ensure that the discharge meets the regulatory guidelines in place (Akcil, 2003).

#### **2.2.1.1 Natural degradation of cyanide**

Natural degradation of cyanide is the process that reduces cyanide concentrations in wastewater systems without human interference. Cyanide undergoes numerous natural

degradation reactions. These reactions include adsorption, biodegradation, oxidation, volatilisation and photodecomposition (Alvarez *et al.*, 2004).

The mechanisms of natural cyanide degradation are dependent on numerous variables such as pH, water chemistry, dissolved oxygen concentration and temperature. The volatilisation of cyanide from the wastewater is mainly dependent on the pH of the solution (Luque-Almagro *et al.*, 2005). Below pH 9, most of the cyanide evaporates as HCN, due to its high vapour pressure. However, with an increase in temperature, reduced liquid-depth to surface-area ratios and turbulence increase the rate of volatilisation significantly (Alvarez *et al.*, 2004; Simovic, 1984). In the presence of air, cyanide is oxidised to form bicarbonate and ammonia as by-products. Increased contact with atmospheric carbon dioxide (CO<sub>2</sub>) with the cyanide-containing solution decreases the pH, in turn increasing the rate of HCN formation, which volatilises more quickly. Ultraviolet rays from the sun facilitate the degradation of metal-complexed cyanides in a process known as photodecomposition. The photodecomposition method is largely effective against strong acid dissociable cyanides such as metallic cyanide complexes in particular iron cyanide complexes. Mostly, the cyanide complexes are highly stable under alkaline conditions but when the pH of the solution is acidic, the solubility is increased, making the complexes mobile and susceptible to photodecomposition. Under these conditions, metal complexes dissociate to their original constituents, free cyanide and the metal ion (Nsimba, 2009, Simovic, 1984).

The majority of the cyanide is degraded through natural decomposition but the metal-complexed cyanides are resistant to degradation owing to the stability between the metal and cyanide molecules. Furthermore, the turbulence of the water can provide pneumatic mixing, thus exposing the metal complexes to sunlight (ultraviolet). This will enhance volatilisation of HCN gas by increasing the surface area and rate of mass transfer (Simovic, 1984). Ultraviolet and visible radiation causes the dissociation of metallic cyanides, especially in the presence of increased dissolved oxygen. However, wastewater containing cyanide and metal cyanides is often found in a stagnant form, limiting exposure of metal cyanides to sunlight, thus their biodegradation. Therefore, it was realised that chemical, physical, and subsequently, biological degradation strategies had to be employed to reduce these limitations (Alvarez *et al.*, 2004).

### **2.2.1.2 Physical and chemical degradation of cyanide**

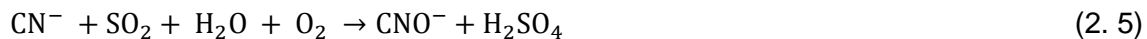
Several methods have been used in the degradation or conversion of cyanides. Some of the predominantly used methods include the barren/fresh water rinse, oxidation using sulphur dioxide (INCO process), and hydrogen peroxide including alkaline chlorination. These



methods represent some of the physical and chemical processes employed to reduce the toxicity of cyanide from cyanide-containing wastewaters.

The barren/fresh water rinse method is mainly based on washing the heap from the barren pond with fresh water, in which the water is also used to reduce evaporative losses. There are no reagents used in this method, and cyanide concentrations decrease owing to the combination of microbial degradation, complexation and volatilisation. This treatment method is best suited to climates that have access to an inexpensive source of fresh water and a negative water balance, to minimise the volume of rinsate generated (Mosher & Figueroa, 1996). This process incurs no additional engineering costs and no additional equipment is required. However, the costs of operation and maintenance are higher as it can take longer to rinse the heap to the required standard. The practicality of this method is therefore virtually impossible, especially in arid regions.

However, the sulphur dioxide (INCO) oxidation is one of the earliest methods employed for cyanide treatment, and was patented in the United States in 1985. The process is able to oxidise both free cyanide (F-CN) and weak acid dissociable cyanides (CN<sub>WAD</sub>) according to the following stoichiometric equation:



The process makes use of SO<sub>2</sub> and air in the presence of a catalyst such as copper to oxidise cyanide to a less toxic form, that is, its cyanate equivalent (Akcil, 2003; Moscher & Figueroa, 1996). However, the metal-complexed cyanides cannot be treated using this process, and the complexes are removed by precipitation. The advantage of using this process is that it is able to treat tailings slurry with varying physicochemical characteristics (Akcil, 2003; Moscher & Figueroa, 1996) and it makes use of inexpensive reagents with one of the main reagents, SO<sub>2</sub>, being a by-product of other processes. On the other hand, the capital costs of this process are higher compared with other chemical processes, due to the complexity of the chemical handling systems required to make the process effective (Moscher & Figueroa, 1996). It is also inefficient in treating strong acid dissociable cyanide (CN<sub>SAD</sub>) and the pH of the solution being treated has to be continuously regulated using lime (CaCO<sub>3</sub>) due to the formation of sulphuric acid. The neutralisation process leads to the formation of excess sludge in a form of metal hydroxides, causing further environmental deterioration (Baxter & Cummings, 2006; Simovic, 1984).

The oxidation of cyanide waste using hydrogen peroxide also facilitates the conversion of these cyanides. The chemistry of this process is similar to that of the INCO process; the difference is that hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is used instead of SO<sub>2</sub>. The treatment of cyanide by H<sub>2</sub>O<sub>2</sub> is shown by the equation below:



A copper catalyst is used to accelerate the conversion of cyanide to cyanate. For this process, H<sub>2</sub>O<sub>2</sub> is supplied in excess and cyanide is converted to cyanate, which in turn dissociates to ammonia and carbon dioxide due to the instability of the cyanate formed. The process is not applied to slurries, because of the high quantities of hydrogen peroxide that are needed to treat the slurry and therefore it is mainly applied to cyanide in solution. This process is pH independent and produces environmentally acceptable effluents without increasing total dissolved solids, as do other chemical processes (Baxter & Cummings 2006; Akcil 2003), but the process has high reagent costs. The new method of mixing hydrogen peroxide and Caro's acid has however reduced the overall reagent costs required for the acquisition of hydrogen peroxide (Moscher & Figueroa, 1996).

Similarly, the alkaline and breakpoint method requires a large quantity of chlorine gas. This method involves a two-step process where cyanide is firstly converted to cyanogen chloride by mixing cyanide and chlorine gas followed by a second step which involves the hydrolysis of the formed cyanogen chloride to form cyanate as described by the equations 2.7 and 2.8 below:



If thiocyanate (SCN<sup>-</sup>) and ammonia are also present in the treated effluent, more chlorine needs to be added to attain the breakpoint of these two compounds, producing nitrogen gas as by-product of the process (Akcil, 2003). The process is unable to treat CN<sub>SAD</sub> but is able to treat CN<sub>WAD</sub> to cyanate. Additionally, thiocyanate is preferentially oxidised to free cyanide, thus increasing the reagent consumption to meet the discharge limits (Baxter & Cummings, 2006). However, this process is out of favour owing to its environmental implications as the reagents (chlorine gas or sodium hypochlorate) that are used in this process have considerable handling problems (Mosher & Figueroa, 1996). Additionally, the chlorination of

cyanides results in the formation of highly toxic intermediates such as cyanogen chloride and other toxic organo-chlorides. Moreover, the process produces high quantities of contaminated sludge which requires licensed disposal, increasing the capital costs of the process (Acheampong *et al.*, 2010).

The above-mentioned chemical processes are commonly utilised processes in industry for treating cyanide-containing effluents. However, these methods have shortcomings that are directly linked to capital costs associated with the implementation of the processes and the production of other intermediate contaminants that further contribute to environmental pollution. Some of these processes produce excess contaminated sludge, which increase to the capital costs associated with the decontamination of the excess sludge. To keep the operating costs as low as possible, cyanide is partially converted to a less toxic cyanate, and in many cases, the non-reacted metal cyanide containing effluents is discharged without further treatment (Acheampong *et al.*, 2010).

### **2.2.2 Biological treatment of cyanide**

The use of microorganisms in treating hazardous substances contaminating the environment has proved to be robust and economically viable (Bitton, 1995). Many microorganisms possess specific enzymes that convert or degrade cyanide compounds by using different biochemical pathways. Several species of bacteria, fungi and algae, utilise cyanide as a source of nitrogen and/or as a carbon source. Cyanide utilisation by these microbial species result in a non-toxic and an environmentally friendly way of degrading the cyanide to less harmful by-products. The biodegradation of cyanide often leads to the formation and accumulation of ammonia and bicarbonate as described in Equation 2.9. For the microorganisms to survive and propagate and be able to degrade cyanide, they require nutrients that are in a form of carbon, nitrogen and phosphorus. Normally, phosphorus is added to the wastewater in a form of concentrated phosphoric acid to concentrations ranging from 1 to 5 mg/L to facilitate the cyanide degradation process (Moscher & Figueroa, 1996).

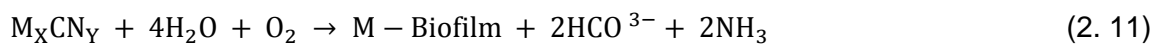


The bicarbonate formed in Equation 2.9 is highly unstable and therefore dissociates to form carbon dioxide or can be utilised by microorganisms as a source of carbon. Often, the biological degradation of cyanide involves two separate processes. In the first stage of the process, cyanide is biologically converted to bicarbonate and ammonia. The decrease in pH

in most of the cyanide biodegradation processes is due to the formation of carbonic acid that results from dissolved carbon dioxide in water as described in Equation 2.10.



The metal-complexed cyanides are also degraded in the first stage of the process where there is subsequent sorption and/or precipitation of metals that are released from the complex (Akcil & Mudder, 2003). The adsorption of metals (indicated as M) during cyanide biodegradation is shown in Equation 2.11.



The treatment of metal-complexed cyanides follows the chemical stability of the metals being treated. Depending on the concentration of cyanide to be treated, the resultant ammonia produced after degradation of cyanide is also of environmental concern and needs to be degraded. The presence of nitrifying and denitrifying bacteria in the second stage of the remediation process promotes the degradation of ammonia in a two-step process. Microbial species from the *Nitrosomas* and *Nitrobacter* genus play a huge role in the nitrification process where the oxidation of ammonia takes place (Equation 2.12), resulting in the formation of nitrite which is an intermediate. The nitrite is then oxidised to nitrate in a rapid second oxidation stage (Equation 2.13) (Akcil *et al.*, 2003).



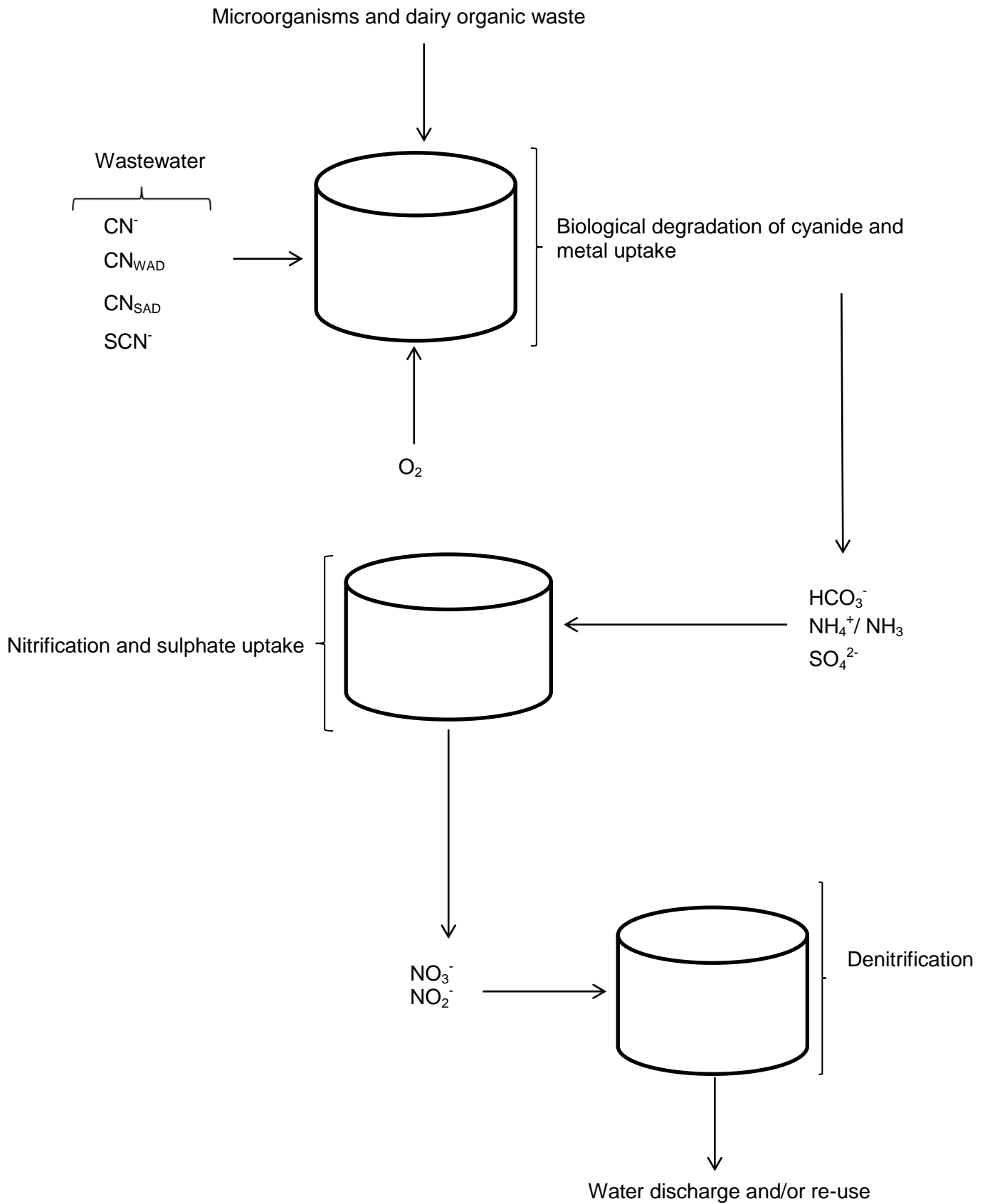
The microbial species in the destruction and nitrification stages are non-competitive and are limited to the presence of cyanide and changes in ammonia concentration. It has however been observed that some of the cyanide-degrading species have the capacity to utilise the ammonium as a nitrogen source, especially in environments where ammonium concentration is higher than that of cyanide (Andrade *et al.*, 1995, Dictor *et al.*, 1997). This has also been observed in environments where the microbial species completely degrades the cyanide and there is an insufficient quantity of the nitrogen source. It is proposed that these species adapt to these conditions and alter their gene structure in order to utilise the

ammonia available as a source of nitrogen. Therefore, these species can serve as cyanide-degrading, nitrifying and denitrifying organisms.

Ammonia serves as a nitrogen source to nitrifying organisms and the bicarbonate serves as a source of carbon. There is therefore no need for nutrient supplementation in the second stage of the biodegradation process. Cyanide is toxic to nitrifying organisms (Kim *et al.*, 2011a, Kim *et al.*, 2011b), and an interruption in the effectiveness of the cyanide degradation process will adversely affect the nitrification stage. The nitrifiers are a rate-limiting step in the plant design for cyanide degradation, due to their slow growth (Akcil, 2003). The capacity of the cyanide-degrading organisms to oxidise ammonia eliminates process disturbances as these species can effectively degrade cyanide and ammonia individually or simultaneously (Andrade *et al.*, 1995, Dictor *et al.*, 1997)

Recent studies have, however, demonstrated the effectiveness of heterotrophic and autotrophic microbial species in nitrification and aerobic denitrification, even at cold temperatures. Shoda & Ishikawa (2014) observed high nitrification and aerobic denitrification ability of *Alcaligenes faecalis* strain no.4 in wastewater containing high concentrations ammonium under heterotrophic conditions and this work corresponded with the results that were obtained by Joo *et al.* (2005). Zou *et al.* (2014) observed nitrification and aerobic denitrification ability of an enriched autotrophic organisms at low temperatures (10 °C) where high nitrogen removal rates were observed. However, there is a lack of information on nitrification and aerobic denitrification in the presence of cyanide using heterotrophs and autotrophs.

A cyanide biodegradation system is proposed as described in Figure 2.2. In the system, the utilisation of agro-waste extracts, in this case, whey waste, as supplements for the microbial species present within the system to support the growth and proliferation of the microorganisms was proposed. This will thus lead to increased cyanide (and related compounds) degradation with subsequent metal biosorption within the biofilm. The proposed three-stage process comprises the cyanide biodegradation and metal biosorption by the microbial species, mostly belonging to the *Bacillus* genus, with subsequent production of ammonium/ammonia and sulphates from the biodegradation of cyanide and thiocyanate, respectively. The application of such waste materials as supplements can thus lead to decreased capital investment while ensuring increased remediation efficiencies. The formed sulphates, ammonia and bicarbonate can be utilised by the organisms as sources of sulphur, nitrogen and carbon, respectively. Denitrification of the formed nitrates takes place within the system, resulting in an effluent that is non-hazardous.



**Figure 2.2:** The mechanisms of aerobic biodegradation of cyanide and subsequent utilisation of the formed by-products, viz ammonium and nitrates.

The advantages and disadvantages of the current cyanide treatment methods are listed on Table 2.4

**Table 2.4:** Advantages and disadvantages of cyanide treatment processes

<b>Cyanide treatment method</b>	<b>Advantages</b>	<b>Disadvantages</b>
Alkaline chlorination	Technology is well established	Excess hypochlorite is toxic Chlorine can react with organic compounds to form chlorinated compounds Reacts preferentially with thiocyanate Adds potentially hazardous cations or anions to water.
Hydrogen peroxide	Excess reagent decomposes to water and oxygen Simple to operate	Reagent is costly Precipitate disposal costs
INCO process	Reagent is cheap	Process adds sulphates to treated water Precipitate disposal costs.
Biodegradation process	Cost effective and robust process Does not generate toxic end products	The technology is not well established

### **2.3 Microorganisms and enzymatic pathways involved in the biodegradation of cyanide**

A number of bacterial species are able to degrade cyanide. An advantage in using these microbial species is that they are adaptable and can be manipulated to fit particular and well-defined bioprocess conditions. Microbial species can thrive under changing conditions, allowing for destruction, sorption and/or uptake of cyanide, heavy metals, ammonia and sulphate. Under stressful conditions, some microbial species are able to produce spores which can germinate when a suitable food source is available. Additionally, some microbial species automatically alter their gene structure as a means of survival in response to the adverse environmental conditions that they are experiencing.

The microbial species that are identified to degrade cyanide comprise the following genera: *Pseudomonas*, *Bacillus*, *Thiobacillus*, *Alcaligenes*, *Bosea*, *Sphingomonas*, *Microbacterium*, *Pussilimonas* and other microorganisms (Makhotla *et al.*, 2010). A list of cyanide-degrading organisms with their target substrates is shown in Table 2.5. These organisms employ different metabolic and enzymatic pathways to degrade cyanide.

**Table 2.5:** Bacterial species capable of degrading cyanide

Microorganism	C-source	N-source	Product(s)	Temp (°C)	pH	Reference
<i>Pseudomonas sp.</i>	Whey	CN <sub>WAD</sub>	NH <sub>4</sub> , CO <sub>2</sub>	30	9.2-11.4	(Akcil <i>et al.</i> , 2003)
<i>Burkholderia cepacia</i> C-3	Glucose, Fructose	KCN, KSCN, Nitriles	NH <sub>3</sub> , HCOOH	30	10	(Adjei & Ohta, 1999)
<i>Azotobacter vinelandii</i>	Cassava	NaCN	NH <sub>3</sub> , CH <sub>4</sub>	30	7-8.5	(Kaewkannetra <i>et al.</i> , 2009)
<i>Klebsiella oxytoca</i>	Glucose	CN, SCN	NH <sub>4</sub> , CH <sub>4</sub>	30	7.0	(Kao <i>et al.</i> , 2003)
<i>Pseudomonas fluorescens</i>	Glucose	Fe(CN) <sub>6</sub>	NH <sub>4</sub> , CO <sub>2</sub>	25	5.0	(Dursun <i>et al.</i> , 1999)
<i>Thiobacillus sp.</i>	CO <sub>2</sub>	SCN	NH <sub>4</sub> , CO <sub>2</sub> , SO <sub>4</sub>	20-40	6-9.3	(Stott <i>et al.</i> , 2001)
<i>Klebsiella sp.</i>	SCN	SCN	NH <sub>4</sub> , SO <sub>4</sub>	38	7.0	(Ahn <i>et al.</i> , 2005)
<i>Halomonas sp.</i>	Glucose, Fructose, CO <sub>2</sub> , Acetate	SCN	NH <sub>4</sub> , CO <sub>2</sub> , SO <sub>4</sub>	20-40	6-9.3	(Stott <i>et al.</i> , 2001)
<i>Pseudomonas pseudoalcaligenes</i>	Acetate	NaCN	NH <sub>4</sub> , CO <sub>2</sub>	30	9.5-10.0	(Huertas <i>et al.</i> , 2010)
<i>Rhodococcus sp.</i>	KCN	KCN	NH <sub>4</sub> , HCOOH	30		(Maniyam <i>et al.</i> , 2013)
<i>Pseudomonas stutzeri</i> , <i>Pseudomonas putida</i>	Lactate, Sucrose	KCN, KSCN	NH <sub>4</sub> , SO <sub>4</sub> <sup>2-</sup> , CO <sub>2</sub>	28-30	9.0-9.2	(Karavaiko <i>et al.</i> , 2000)



There is lack of literature on the characterisation of cyanide-degrading microorganisms and this is a limitation to understanding the processes in which these microorganisms are used and the development of a predictive operational model for such processes (Stott *et al.*, 2001). The Homestake mine cyanide treatment process in the USA showed the importance of understanding the microbial population in a cyanide-degrading process. The initial assessment that was employed using continuous stirred tank reactors (CSTRs) showed unfavourable results, owing to limited knowledge and understanding of the microbial population used. Subsequent work using CSTRs and rotating biological contactors (RBCs) with a different microbial consortium showed encouraging results for cyanide degradation (Stott *et al.*, 2001). The oxygen concentration has been found to have an effect on the growth and proliferation of the microbial species. Previous work has shown that the growth of *Pseudomonas fluorescens* on the degradation of ferrocyanide was reduced due to limited oxygen in the system (Dursun *et al.*, 1999). However, it has been shown that to prevent volatilisation of cyanide as HCN gas, dissolved oxygen concentrations should not exceed 2 mg/L in the wastewater to be treated (Kaewkannetra *et al.*, 2009). Similarly, the pH has been shown to significantly influence the microbial activity over a pH of 9.5 to 10.0. When the pH was reduced from 10.0 to 9.5, the lag phase of *Pseudomonas pseudoalcaligenes* significantly decreased (Huertas *et al.*, 2010).

Research has been done on cyanide degradation using a *Bacillus pumilus* strain. The addition of trace elements such as Manganese (Mn) at low concentrations was found to significantly increase the biodegradation of cyanide. Additionally, the use of tap water rather than distilled water was suggested, as the *Bacillus* species degraded cyanide more successfully in a medium prepared with tap water than in a medium prepared with distilled water. This was due to the presence of trace elements in tap water (Meyers *et al.*, 1991).

Desirable operational parameters such as temperature, influent cyanide concentration and aeration should be considered, as these external factors contribute to the type of degradation pathway employed by the microorganisms (Dash *et al.*, 2009). For example, the presence of cyanide, either as free cyanide or complexed cyanide, can result in the activation of one or two enzymatic pathways for the degradation of cyanide, depending on the microbial species employed in such a process and environmental conditions (Ebbs, 2004). The solubility and bioavailability of cyanide in soil-water systems can also influence the selection of a desired pathway by the microorganisms (Dash *et al.*, 2009).

The degradation of cyanide is not only limited to bacterial species. A number of fungal and algal species have been isolated from cyanide wastewater and other similar environments.

These microbial species have been found to degrade cyanide. *Scenedesmus obliquus* was fed with 78 mg CN<sub>WAD</sub>/L in a batch system where it was able to degrade the cyanide to less than 6 mg CN<sub>WAD</sub>/L in 72 hours. The algal species was also able to sequester elevated levels of metals (Gurbuz *et al.*, 2009). However, the research conducted by Gurbuz *et al.* (2009) did not contain a reference experiment (control) for comparison purposes, since cyanide is known for its high volatility. Therefore, the cyanide attributed to biodegradation by the algal species could be due to volatilisation of cyanide and not biodegradation. In another study, *Acremonium strictum* was able to degrade up to 7400 mg SCN<sup>-</sup>/L within 85 hours. This fungal species is one of the few fungi that have been reported to degrade higher concentrations of thiocyanate (Kwon *et al.*, 2002).

The ability of the *Trichoderma* species to degrade free cyanide was investigated, in comparison with *Furasium* species, which is known to degrade free cyanide. In one experiment, cyanide was used as a sole carbon and nitrogen source; in the other, glucose was supplemented in fed-batch mode. The organism was able to degrade 2000 CN<sup>-</sup>/L within 90 days without glucose supplementation. When glucose was used as a supplement, the organisms were able to degrade 2000 mg F-CN/L within 32 days (Ezzi & Lynch, 2005). These results showed the importance of adding carbon sources to cultures in which microbial species are used to degrade cyanide, as it enhances the growth of the microbial species, thus increasing contaminant degradation rates. However, the research conducted did not contain any control for comparison purposes; therefore, the cyanide claimed to have been biodegraded might have been due to volatilisation.

Work on free cyanide biodegradation using *Aspergillus awamori* (*A. awamori*), a new fungal strain that is structurally and biochemically similar to *Aspergillus niger*, has been investigated. It was shown that the fungal strain can tolerate up to 430 mg F-CN/L and was able to degrade cyanide at concentration between 150 mg F-CN/L and 250 mg F-CN/L (Santos *et al.*, 2013a). Table 2.6 lists some of the fungal and algal species that are able to degrade cyanide.

**Table 2.6:** Fungal and algal species capable of degrading cyanide

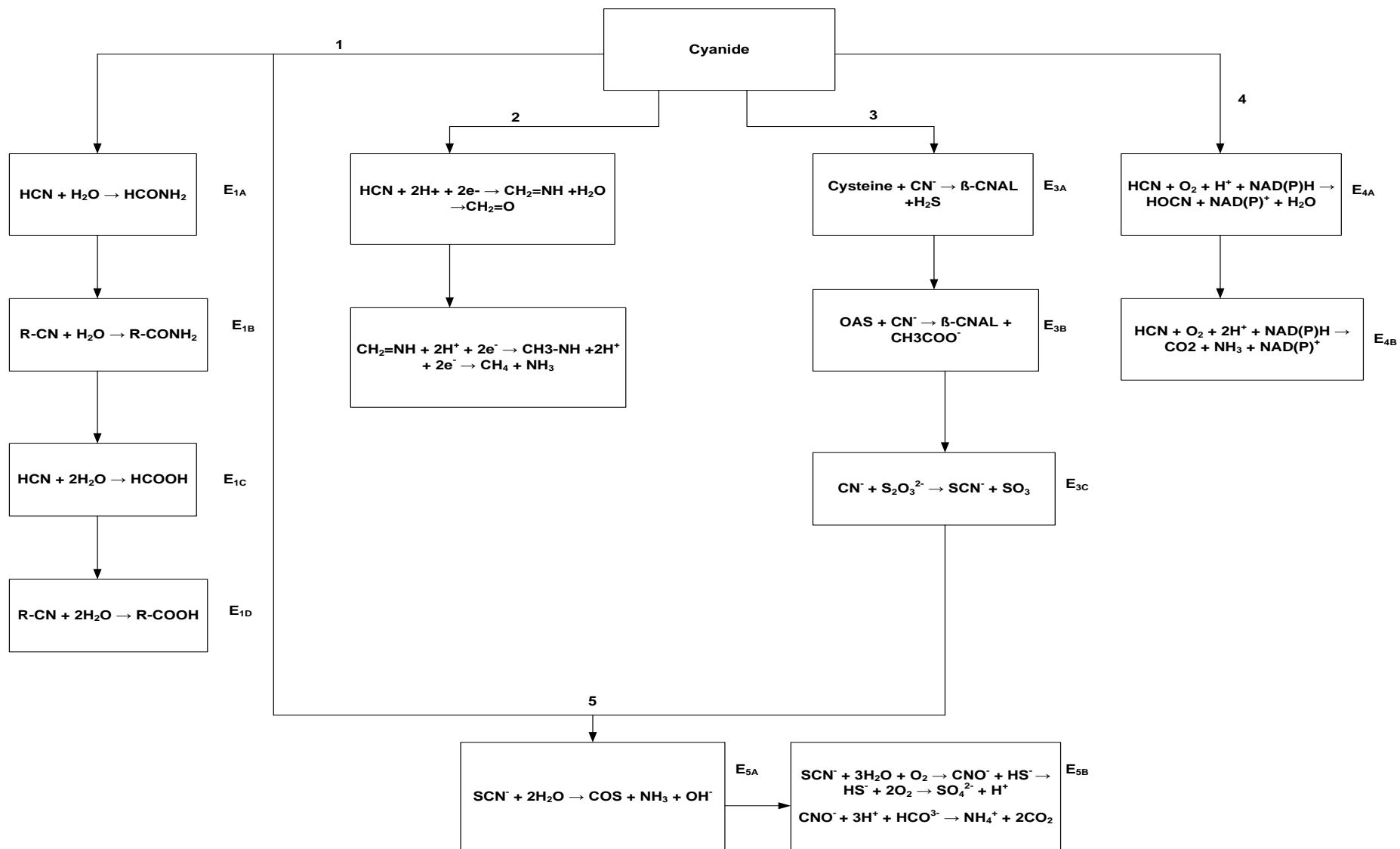
Microorganism	C-source	N-source	Products	Temperature (°C)	pH	Reference
<i>Fusarium solani</i>	Glucose	K <sub>2</sub> Ni(CN) <sub>4</sub> , KCN	NH <sub>4</sub> , HCOOH	25	7.0	(Barclay <i>et al.</i> , 1998)
<i>Scenedesmus obliquus</i>	NaCN	NaCN	NH <sub>4</sub> , CO <sub>2</sub>	-	10.3	Gurbuz <i>et al.</i> , 2009
<i>Aspergillus awamori</i>	<i>Citrus sinensis</i> extract	KCN	NH <sub>4</sub> , HCOOH	40	8.84	(Santos <i>et al.</i> , 2013a, Santos <i>et al.</i> , 2013c)
<i>Trametes versicolor</i>	Citrate	KCN	NH <sub>4</sub> , CO <sub>2</sub>	30	10.5	(Cabuk <i>et al.</i> , 2006)
<i>Trichoderma ssp.</i>	Glucose	CN <sup>-</sup>	NH <sub>4</sub> , CO <sub>2</sub>	25	6.5	(Ezzi & Lynch, 2005)

### **2.3.1 Degradation pathways for cyanide and cyano-group containing compounds**

Microbial species employ different biochemical pathways in response to the environmental stress they experience. Sometimes, the same microorganism can employ different biochemical pathways for the degradation of cyanide. Five enzymatic pathways for the destruction of cyanide and cyano-group containing compounds have been identified (Dash *et al.*, 2009, Ebbs, 2004). These are:

- Oxidative pathway
- Reductive pathway
- Hydrolytic pathway
- Substitution or transfer pathway
- Synthesis pathway

The first three pathways are degradation pathways where microbial enzymes catalyse the degradation of cyanide to ammonia, CO<sub>2</sub>, methane, formic acid and carboxylic acid. The last two pathways are assimilative pathways. The type of enzymatic pathway that the microbial species employ is dictated by the environmental conditions (pH, temperature and presence of toxins) and concentration of cyanide. All the enzymatic reactions involved in the successful bioremediation of cyanide are shown in Figure 2.3.



**Figure 2.3:** Enzymatic reactions involved in cyanide biodegradation. 1- Hydrolytic reactions; 2- Reduction reactions; 3- Substitution reactions; 4- Oxidation reactions; 5- Thiocyanate biodegradation;  $E_{1A}$ - Cyanide hydratase;  $E_{1B}$ - Nitrile hydratase;  $E_{1C}$ - Cyanidase;  $E_{1D}$  – Nitrilase;  $E_{3A, 3B}$  – Cyanoalanine synthase;  $E_{3C}$  – Thiosulfate: cyanide transferase;  $E_{4A}$  – Cyanide monooxygenase;  $E_{4B}$  – Cyanide dioxygenase;  $E_{5A}$  – Thiocyanate hydratase ;  $E_{5B}$  - Cyanase

### 2.3.1.1 Oxidative pathway

The degradation of cyanide via the oxidative reactions leads to the formation of ammonia and carbon dioxide. This pathway requires NADPH as well as an additional carbon source. There are two distinctive oxidative pathways, with reactions catalysed by two different enzymes. The first pathway converts cyanide to cyanate, with cyanase then catalysing the bicarbonate-dependent conversion of cyanate to ammonia and carbon dioxide. Cyanases have been identified in numerous bacteria, fungi, plants and animals. The role of cyanase has been presumed to be a protection mechanism against cyanate poisoning (Dash *et al.*, 2009, Ebbs, 2004). The second pathway directly converts cyanide to ammonia and carbon dioxide in a reaction that is catalysed by cyanide dioxygenase. It has been proposed that this pathway is dependent on the cofactor protein and the formation of cyanohydrins as an important step for cyanide degradation via the oxygenase-mediated pathways (Ebbs, 2004).

*Pseudomonas putida* utilises the oxidative pathway for the degradation of cyanide. This microorganism is well suited to industrial applications as it is able to survive at high pH up to 11.5 in 780 mg F-CN/L cyanide (Chapatwala *et al.*, 1998). *P. putida* was able to degrade thiocyanate, resulting in the formation of sulphite as well as sulphate, tetrathionate, trithionate and thiosulphate, a characteristic mainly attributed to the availability of dissolved oxygen (Luque-Almagro *et al.*, 2005; Chapatwala *et al.*, 1998; Gupta *et al.*, 2010; Dash *et al.*, 2009).

### 2.3.1.2 Reductive pathway

There is limited literature on the cyanide-degrading enzymes that utilise this pathway. However, *Klebsiella oxytoca* has been shown to degrade cyanide via the reductive pathway, in which cyanide is ultimately converted to methane and ammonia (Chen *et al.*, 2008, Kao *et al.*, 2003).

### 2.3.1.3 Hydrolytic pathway

The hydrolytic mechanism for cyanide degradation offers many advantages. The hydrolytic enzymes directly cleave the triple bond between the carbon and nitrogen atom, destroying the cyano moiety. Biological degradation of cyanide via the hydrolytic pathway does not rely on the viability and activity of cells for cyanide detoxification (Raybuck, 1992). To date, there are five enzymes that utilise the hydrolytic pathway to degrade free cyanide and nitriles. Cyanidase, cyanide hydratase, nitrilase, nitrile hydratase and thiocyanate hydrolase have been identified as the main enzymes in the direct conversion of cyanide and nitriles to carbon dioxide and ammonia, with formamide being an intermediate of the reaction (Gupta *et al.*, 2010). Cyanide hydratase is mainly of fungal origin and cyanidase is mainly bacterial. These

two enzymes are highly specific to cyanide as a substrate. Nitrilase and nitrile hydratase are mainly involved in the degradation of nitriles. These two enzymes are specific to their substrate but their specificity is lower than that of cyanidase and cyanide hydratase. Nitrilase and nitrile hydratase are responsible for the conversion of aliphatic and aromatic nitriles to carboxylic acid and formamide, with the formamide being converted to ammonia through the action of an amidase enzyme.

The biodegradation of thiocyanate is mainly catalysed by thiocyanate hydrolase to form sulphate, ammonia and carbon dioxide. The thiocyanate hydrolase mainly acts on carbon-nitrogen bonds other than peptide bonds, specifically for thiocyanate degradation. This is the case with *Thiobacillus thioparus*, which employs the thiocyanate hydrolase to degrade thiocyanate, producing carbonyl sulphide (COS) and ammonia. The COS produced is reduced to hydrogen sulphide (H<sub>2</sub>S) and CO<sub>2</sub>. These by-products, in combination with ammonia, serve as energy, sulphur and nitrogen sources for microbial growth (Gupta *et al.*, 2010).

#### **2.3.1.4 Substitution or transfer pathway**

The first of the assimilative pathways is the transfer pathway. In this pathway, a transfer of sulphur species from a source (normally thiosulfate) is donated to the cyanide ion, thereby forming thiocyanate. This pathway involves cyanide assimilation with subsequent microbial growth due to the provision of an extra nitrogen source. The enzymes that mainly catalyse the transfer of the sulphur species belong to the sulfurtransferase family. Two enzymes are of particular interest in this pathway: rhodanese and mercaptopyruvate sulfurtransferase (Gupta *et al.*, 2010; Dash *et al.*, 2009; Bhalla *et al.*, 2012).

Rhodanese enzymes are highly conserved and are currently regarded as one of the mechanisms evolved for cyanide degradation in various species. The activity of the enzyme is regulated by phosphate ions and divalent ions that interact with the active site of the enzyme (Gupta *et al.*, 2010). The rhodanese enzymes are able to function at their optimum at temperatures between 35 to 55°C and at a pH range of 8.5 to 11.5.

The mercaptopyruvate sulfurtransferase enzyme catalyses a two-step reaction whereby an enzyme-sulphur intermediate is formed in the first reaction. The intermediate further reacts with free cyanide and the enzyme transfers the sulphur moiety to the cyanide ion, thus forming thiocyanate as an end product (Gupta *et al.*, 2010). The thiocyanate that is formed in this pathway can be further degraded via the hydrolytic pathway by thiocyanate hydratase.

### 2.3.1.5 Synthesis pathway

The synthesis pathway is an assimilative pathway and normally results in the synthesis of amino acids such as  $\beta$ -cyanoalanine and  $\gamma$ -cyano- $\alpha$ -aminobutyric acid by using amino acid precursors to react with cyanide. The  $\beta$ -cyanoalanine and  $\gamma$ -cyano- $\alpha$ -aminobutyric acid are structurally homologous to each other (Gupta *et al.*, 2010). The synthesis of amino acids from cyanide is catalysed by two enzymes, mainly cyanoalanine synthase and  $\gamma$ -cyano- $\alpha$ -aminobutyric acid synthase. The cyanoalanine synthase belongs to the lyase enzyme family and catalyses the reaction between free cyanide and L-cysteine to form  $\beta$ -cyanoalanine and hydrogen sulphide as products (Gupta *et al.*, 2010). During this process, there is no direct requirement for oxygen or NADPH, nor is carbon dioxide produced (Dash *et al.*, 2009). The  $\gamma$ -cyano- $\alpha$ -aminobutyric acid synthase requires phosphate to function and it is induced by glutamate or glycine. Once the  $\gamma$ -cyano- $\alpha$ -aminobutyric acid is synthesised, it is slowly converted to glutamate, an amino acid.

### 2.3.2 Degradation of nitriles and metal-complexed cyanides

Cyanide is able to form complexes with many chemical compounds, both organic and inorganic. Nitriles are produced when hydrogen cyanide donates the cyano-group to an organic compound, forming a nitrile such as acetonitrile.

#### 2.3.2.1 Biodegradation of nitriles

Nitriles are defined as cyanide-substituted carboxylic acids. Nitriles occur in nature and some are produced by microorganisms (Kao *et al.*, 2006). Nitrile metabolising enzymes are found in bacterial, fungal and plant species. Aliphatic and aromatic nitriles are transformed by nitrilases and nitrile hydratases to corresponding carboxylic acid and amide, respectively. Some microbial species are able to transform free cyanide to nitriles, such as  $\beta$ -cyano-L-alanine, and degrade these nitriles to corresponding carboxylic acid and amides through action of nitrilases, nitrile hydratases and amidases (Bhalla *et al.*, 2012). It has been suggested that some fungal species are able to use the produced carboxylic acid as an additional source of carbon (Santos *et al.*, 2013c).

*Klebsiella oxytoca* was evaluated for its capability to degrade a range of nitrile compounds. It was found that succinonitrile and valeronitrile were the most optimal nitrogen sources for the growth of the organism. Kao *et al.* (2006) found that the degradation of acetonitrile and propionitrile was a two-step process, from acetamide, followed by the formation of acetic acid. The potential applicability of yeast for the degradation of acetonitrile was evaluated using *Candida guilliermondii* and it was found that the yeast was capable of degrading



acetonitrile in a two-step process leading to production of acetic acid and ammonia as end-products (Dias *et al.*, 2001).

#### **2.4 Biodegradation of cyanide using biofilm systems**

Biofilms are clusters of microorganisms that are attached to a carrier or surface (Singh *et al.*, 2006). Microbial species in the environment form biofilms as a protection mechanism from adverse environmental conditions. Substances such as extracellular polymeric substances secreted by these organisms assist these microorganisms in their attachment to surfaces (Africa, 2009, Bitton, 1995, Singh *et al.*, 2006). The biofilm provides sufficient nutrients to the organisms and creates a more tolerable environment, allowing the organisms to grow maximally. Additionally, biofilms have anionic properties, mainly due to the presence of uronic acids within their agglomerated biomass, which enhance the entrapment of cationic species such as transition metals (Cu, Ni, Fe, etc.) on to their surface, thus removing these metals from solution through biosorption (Singh *et al.*, 2006).

Biological treatment of cyanide containing wastes with fixed-film processes has been previously demonstrated using both trickling filters and rotating biological contactors (RBC). Evangelho *et al.* (2001) investigated the biological treatment of free cyanide and thiocyanate from gold mill effluents using a trickling filter reactor. They found that 90% of cyanide, thiocyanate and metals (Cu, Fe & Ni) were removed without a recycle stream (Evangelho *et al.*, 2001). Furthermore, Jeong and Chung (2006) used fluidised carriers as microbial attachment support for the bioremediation of thiocyanate, for which 99.9% removal efficiency was achieved in 36 hours using a feed containing 7000 mg/L of thiocyanate (Jeong & Chung, 2006).

Rotating biological contactors (RBCs) have been successfully applied to laboratory-based studies on the remediation of silver complexed cyanides derived from the electroplating industry (Patil & Paknikar, 2000). A commercial application of the RBCs for treatment of mine effluents has also been applied elsewhere (Baxter & Cummings, 2006), involving the use of a large quantity of RBCs in series to process 870 m<sup>3</sup>/hour with a cyanide-removal efficiency of 95% (Ebbs, 2004; Akcil, 2003).

Bioreactors in series with a sludge recycle unit attached to these reactors were employed in the biological destruction of thiocyanate at different concentrations in continuously stirred tank reactors. Complete thiocyanate degradation occurred at concentration below 200 mg SCN<sup>-</sup>/L, while above this concentration, the immobilised microbial community could not completely degrade thiocyanate at residence times up to 8 hours. However, if the residence time was increased, complete degradation of thiocyanate would be observed. This shows the

importance of correctly adjusting operational parameters for an efficient biodegradation process (Van Zyl *et al.*, 2011).

The tolerance and biodegradation capability of methanogenic microorganisms to free cyanide in an upflow anaerobic sludge blanket (UASB) reactor was evaluated. It was observed the methogens were tolerant and able to degrade free cyanide at concentrations up to 125 mg CN<sup>-</sup>/L. However, it was also shown that the presence of cyanide affected the production of methane gas. This shows the efficiency of microbial species in biofilms, even to microorganisms that are known to be sensitive to low cyanide concentrations when in the planktonic phase (Gijzen *et al.*, 2000).

## **2.5 Biotechnological application of agricultural waste as primary supplements in cyanide degrading bioprocesses**

The agricultural and food industries produce over a billion tonnes of agro-waste around the world each year. These materials are mostly disposed of as waste and/or sometimes used as feedstock for animal feed. There is currently extensive research on the application of these waste products for production of value-added products. Agro-waste materials are being utilised for the extraction of antioxidants from waste peels (Rehman *et al.*, 2004), remediation of heavy metals using the agro-waste as adsorbents (Dakiky *et al.*, 2002, Mohan *et al.*, 2006, Sarin & Pant, 2006, Sharma & Bhattacharyya, 2005, Tarley *et al.*, 2004) and biological production of value-added biotechnological products (Mahmood *et al.*, 1998, Plessas *et al.*, 2007).

The biotechnological application of such waste materials for the remediation of environmental contaminants is growing. The utilisation of waste materials as potential supplements in bioprocesses is mainly due to the presence of organic carbon sources and trace elements such as manganese and magnesium that can facilitate the growth of microbial species (Tran & Mitchell, 1995), in this case, for the degradation of environmental contaminants. Therefore, the application of such waste materials in bioprocesses can add value and lower capital and operational costs, as there will be no need to supplement the cultures with refined carbon sources and/or other macro including micro-nutrients (Christensen *et al.*, 2011). The agro-waste materials that have the potential to be used as supplements in wastewater treatment are listed in Table 2.7.

**Table 2.7:** Current and potential carbon sources used in bioprocesses (Vandamme, 2009)

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<b>Carbohydrates:</b>	
Whey	Cane molasses
Starch (from plant materials)	Beet molasses
Lactose	Sulphite waste liquor
Glucose syrups (from starch saccharification)	Wood hydrolysate
Dextrose	Organic acids
Dextrin	Agro-waste
Corn flour	

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<b>Oils and alcohols:</b>	
Methyloleate, corn oil, soybean oil, palm oil	Glycerol
Fish oil, cottonseed oil, peanut oil	Ethanol
	Methanol
	Hydrocarbons

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The application of *Citrus sinensis* (*C. sinensis*) as pseudo-catalyst for the conversion of free cyanide was evaluated. Santos *et al.* (2013b) observed that the hydrolysed orange peel was more efficient than the unhydrolysed peel, with 63% and 18% cyanide conversion for the hydrolysed and unhydrolysed orange peel, respectively. However, the researchers found that the presence of metals (Cu, Ni, Zn, etc.) inhibited cyanide conversion, because of competition between the metals and cyano-group for the hydroxyl groups present within the orange peel.

The application of agro-waste materials as primary substrates for the biodegradation of F-CN by *Aspergillus awamori* (*A. awamori*) was evaluated. The authors found that *A. awamori* degraded F-CN efficiently in a medium supplemented with orange (*C. sinensis*), followed by carrot (*D. carota*), onion (*A. cepa*) and apple (*M. pumila*) (Ntwampe & Santos, 2013). In another study, a dual-stage membrane bioreactor was utilised in the continuous bioremediation of electroplated wastewater. The dual-stage reactor consisted of a primary stage containing *C. sinensis* pomace and a secondary stage that contained active *A. Awamori* biomass that was supplemented with *C. sinensis* extract. Generally, the authors observed degradation and absorption/adsorption efficiencies of over 99% for cyanide and heavy metals in repeated cycle (n = 3) was achieved. The *C. sinensis* pomace was found to degrade and adsorb the cyanide and metals respectively, even after 3 regeneration cycles, thus highlighting the potential reusability of the pomace for industrial remediation of cyanide wastewater (Santos *et al.*, 2013c)

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# **CHAPTER 3**

## **MATERIALS AND METHODS**

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## CHAPTER 3 MATERIALS AND METHODS

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### Materials and methods

This chapter presents a description of the materials, reactor setup, experimental protocols and analytical procedures used in this study. The rationale for the use of particular materials and methods is also discussed in this chapter.

### 3.1 Wastewater collection and composition

Wastewater samples were collected from an electroplating plant located in Cape Town, Western Cape, South Africa. Previous studies conducted by Santos *et al.* (2013c) have demonstrated that electroplating wastewater is highly contaminated with high concentrations of F-CN ( $\geq 150$  mg F-CN/L) and metal-complexed cyanides. The cyanide-containing wastewater was obtained from randomly selected sites from an electroplating facility. The obtained wastewater was stored in non-transparent 20 L bottles and immediately stored at 4°C to prevent ultraviolet rays (sunlight) from degrading cyanide in the collected samples. The composition of the wastewater as detected by Santos *et al.* (2013c) is described in Table 3.1.

**Table 3.1:** Composition of the electroplating wastewater (adopted from Santos *et al.*, 2013c).

Parameters	Electroplating wastewater
Temperature	22.5 ( $\pm$ 12.50) °C
pH	10.46 ( $\pm$ 0.88)
Total cyanide as CN	149.11 ( $\pm$ 50.75) mg/L
Total ammonia as N	nd
Copper as Cu	45.19 ( $\pm$ 25.89) mg/L
Zinc as Zn	9.05 ( $\pm$ 5.26) mg/L
Nickel as Ni	8.12 ( $\pm$ 4.78) mg/L

nd – Not detected

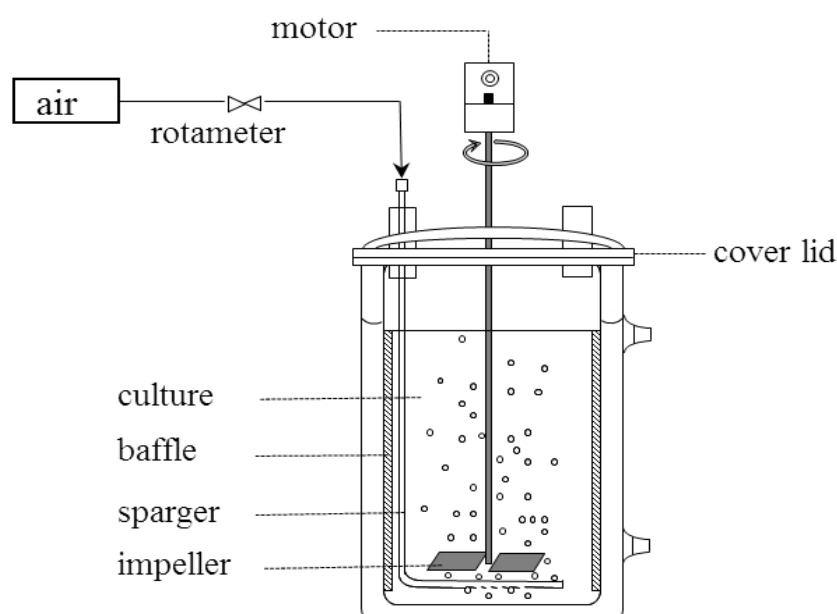
## 3.2 Microbial cultures

### 3.2.1 Isolation of the bacterial species

The wastewater (1L) was filtered using a 0.22  $\mu\text{m}$  Millipore™ membrane filter and the bacterial cake on the filter was suspended in phosphate buffer (pH 7). Serial dilutions were prepared using sterile distilled water followed by plating on nutrient agar plates containing 200 mg F-CN/L as KCN and cyclohexamine (to prevent fungal growth), with subsequent incubation at 37 °C for 48 hours. Colonies were selected based on morphological identification, that is, persistent growth in cyanide-containing media, and transferred to nutrient agar to isolate single colonies. The isolated cultures were continuously maintained on nutrient agar slants and stored at 4 °C.

### 3.2.2 Activated sludge or ammonia-oxidising bacteria (AOB)

Activated sludge was obtained from a wastewater treatment plant (WWTP) in the Western Cape Province, South Africa. The sludge was maintained in a stirred tank reactor with a maximum volume of one litre. Aeration was set to 0.4L/min and the system was operated at room temperature (24 to 27 °C). Mixing was achieved using an overhead stirrer fitted with a four-bladed impeller, set at 250 rpm. The sludge was sub-cultured twice a week using a phosphate buffer (pH 7.00  $\pm$  0.02) that contained NaHCO<sub>3</sub> (4 g/L) as a source of carbon, and ammonium (200 mg NH<sub>4</sub><sup>+</sup>-N/L) as a source of nitrogen. This culture was used as an inoculum for the combined nitrification and aerobic denitrification experiments.



**Figure 3.1:** The schematic diagram of a stirred tank reactor setup for the maintenance of the AOB

The sodium bicarbonate (NaHCO<sub>3</sub>) was used as a carbon source for the AOB throughout the experimental runs.

### 3.3 Cryopreservation of the microbial cultures

The microbial cultures were preserved according to the method developed by Perry (1998), whereby a sterile glycerol solution (40 % v/v) was used to preserve the cultures. Single colonies were inoculated in nutrient broth and grown for 24 hours at 37 °C. The cells were diluted in a 1:1 (v/v) ratio with the sterile glycerol in cryogenic tubes and stored at -80 °C until further use.

### 3.4 Media preparation and culture conditions for cyanide biodegradation using isolated bacterial species

The isolated bacterial species (n = 13), referred to as cyanide degrading bacteria (CDB), were mixed and cultured in an Oxoid™ nutrient broth for 48 hours at 37 °C using a rotary shaker at 180 rpm. This culture was used as an inoculum at a cell concentration of 10% (v/v) per batch for the biodegradation of cyanide. Initially, experiments were conducted in batch systems with F-CN concentrations of 200, 300, 400 and 500 mg F-CN/L on nutrient broth, in order to assess the viability of the cultures to effectively biodegrade cyanide over a period of eight days. In order to effectively assess cyanide tolerance and culture viability on cyanide biodegradation, the isolated microbial species were evaluated in cultures containing alternative carbon sources, that is, agro-waste [*Ananas comosus* (pineapple) extract (1% v/v), *Ipomoea batatas* (sweet potato) extract (1% v/v) and *Beta vulgaris* (beetroot) extract (1% v/v)], spent brewer's yeast (SBY) extract (1% v/v) and whey (0.5 w/v) as sole nutrient and energy sources for the microorganisms. The cultures contained 200 and 400 mg F-CN/L, respectively and the experiment was ran for a period of five days. All the experiments had a final volume of 100 mL using airtight, multiport, round-bottomed flasks with a sampling port while the pH was kept at 9.5 in a phosphate-buffered medium. Potassium cyanide (KCN) was used as a source of F-CN. The biodegraded cyanide, taking into account cyanide volatilisation, was calculated using Equation 3.1 and 3.2.

$$CN_{S}^{-} - (CN_{R}^{-} + CN_{V}^{-}) = CN_{B}^{-} \quad (3.1)$$

where

$$CN_{V}^{-} = (CN_{V_o}^{-} - CN_{V_f}^{-}) \quad (3.2)$$

Where  $CN_{S}^{-}$  was the initial F-CN concentration in the fermentation broth (mg/L),  $CN_{R}^{-}$  was the residual F-CN measured in the cultures (mg/L),  $CN_{V}^{-}$  was the cyanide that volatilised during culture incubation (mg/L),  $CN_{B}^{-}$  was the cyanide that was biodegraded (mg/L),  $CN_{V_o}^{-}$  was the initial cyanide concentration in the control cultures (mg/L), and  $CN_{V_f}^{-}$  was the final cyanide concentration in the control cultures (mg/L).

### 3.5 Preparation of agricultural waste extracts

Agro-waste extracts were prepared by drying the agro-waste at 80 °C for seven days followed by milling to less than 100 µm. The dried waste was mixed with water and autoclaved at 116 °C for 20 minutes. The solution was filtered through a 0.22µm Millipore™ filter and the debri-free extract was used for the experiments at the required concentration.

### 3.6 Response surface methodology: central composite design

Response surface methodology (RSM) is a modelling technique used to assess a relationship between a set of controllable experimental factors and observed results. The optimisation process involves three major steps: (i) performing statistically designed experiments, (ii) estimation of the coefficients in a mathematical model, and (iii) predicting the response and checking the adequacy of the model (Box & Hunter, 1957). In this study, the central composite design (CCD) was used to generate a 15-run experimental plan and the independent variables (temperature, pH and whey concentration) were studied at five different levels coded as  $-\alpha$ , -1, 0, +1 and  $+\alpha$ , representing a core factorial, centre and axial points (Table 3.2). The experimental design used in this study in coded values is tabulated in Table 3.3. The experiment was conducted for a period of 96 hours, in multiple-port airtight shake flasks, to prevent volatilisation of cyanide as HCN gas where the flasks were inoculated with 10 % (v/v) inoculum size from a previously grown culture while the uninoculated flasks served as a control at a cyanide concentration of 500 mg F-CN/L. The pH of the media was adjusted using 1M NaOH or 1M HCl. All the runs were conducted in duplicate and the average of cyanide biodegradation obtained from the data was taken as the dependent variable or response (Y). The second-order polynomial coefficients were calculated and analysed using Design-Expert® Software (version 6.0.8, Stat-Ease Inc., Minneapolis, USA). The behaviour of the system can be described using the following equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + \varepsilon \quad (3.3)$$

where  $Y$ ,  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$  and  $\beta_{ij}$  represent response variable, interception coefficient, coefficient of linear effect, coefficient of quadratic coefficient, and interaction coefficient, respectively.  $X_i$  and  $X_j$  are input variables that influence the response ( $Y$ ), while  $\varepsilon$  represents the random error.

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included the Fisher's  $F$ -test (overall model significance), its



associated probability  $p(F)$ , correlation coefficient  $R$ , and determination coefficient  $R^2$ , which measures the goodness of fit of regression model.

**Table 3.2:** The various media components included in CCD experiments and their corresponding high, medium and low concentration levels

Variables	Units	Code	High levels (+1)	Medium levels (0)	Low levels (-1)
pH	-	A	11.00	10.25	9.50
Substrate concentration	g/L	B	15.00	12.50	10.00
Temperature	°C	C	40.00	32.50	25.00

$\alpha = 1.682$

**Table 3.3:** Central composite design for three variables (coded values)

Run	A	B	C
1	-1	-1	+1
2	-1	+1	+1
3	+1	-1	-1
4	+1	-1	+1
5	0	0	0
6	0	0	+ $\alpha$
7	+1	+1	-1
8	+ $\alpha$	0	0
9	-1	+1	-1
10	+1	+1	+1
11	0	0	- $\alpha$
12	0	- $\alpha$	0
13	0	+ $\alpha$	0
14	-1	-1	-1
15	- $\alpha$	0	0

A, B and C represent the coded level of variables while  $\alpha$  represents the axial point with coded level of 1.682

### 3.7 Nitrification studies using the isolated microorganisms (CDB) and the AOB

#### 3.7.1 Inoculum preparation

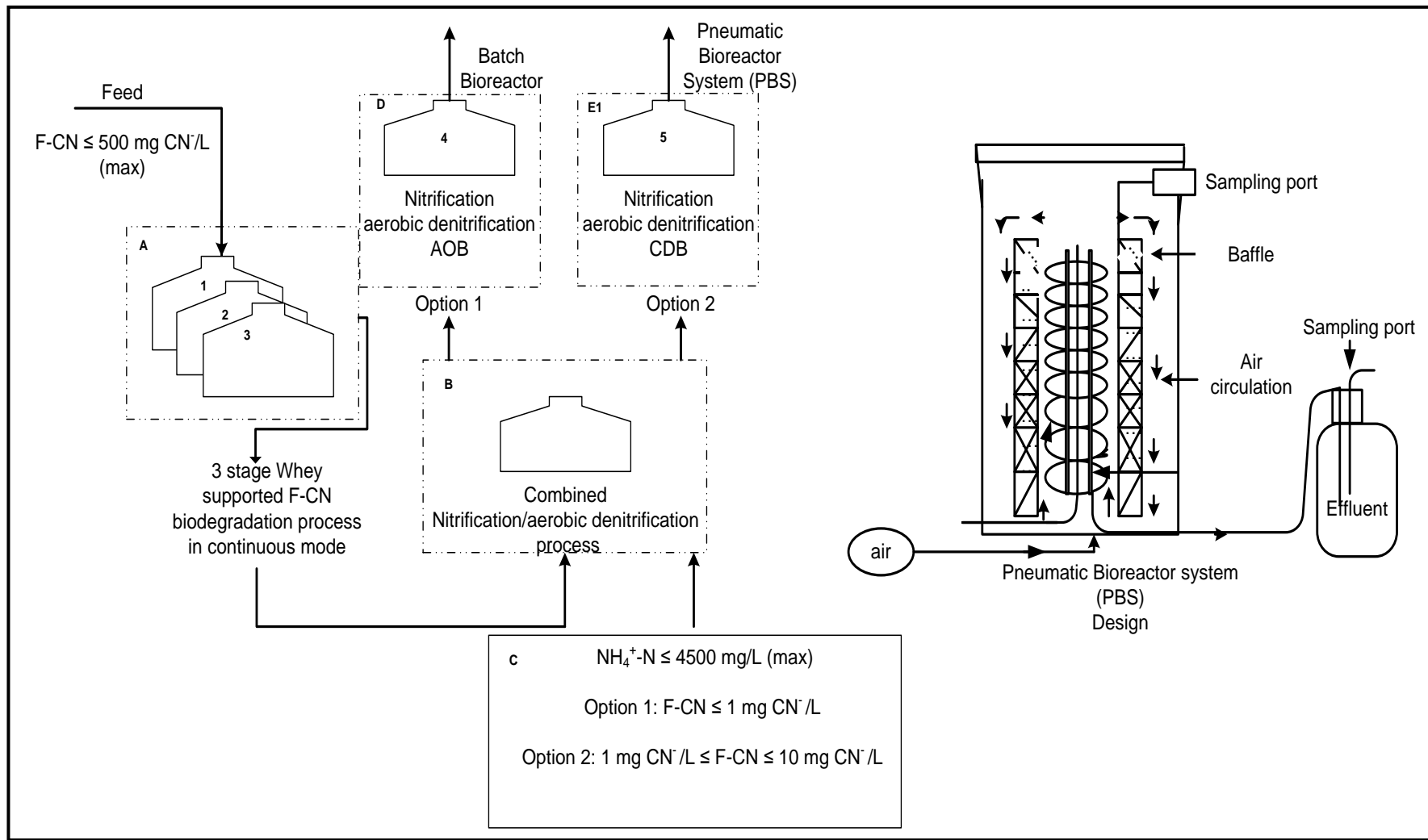
Thirteen pure bacterial isolates were inoculated in a phosphate buffered medium containing 8 g/L of whey, without the addition of a nitrogen source. These bacterial species were isolated from cyanide containing wastewater and were found to be cyanide degraders. The mixed consortia were incubated in a shaking incubator at a temperature of 37 °C for 48 hours with a shaking speed of 180 rpm. This culture was used as an inoculum for the combined nitrification and aerobic denitrification experiments.

The inoculum for the AOB was obtained from the stirred tank reactor as described in Section 3.2.2. The inoculum concentration was set at 10 % (v/v) for the nitrification and aerobic denitrification study using  $\text{NaHCO}_3$  as the carbon source for the autotrophic organisms.

### **3.7.2 Bioreactor setup and operation**

The medium was prepared in a phosphate buffer using whey (15 g/L). Batch experiments (BCs) were carried out in 500 mL Erlenmeyer flasks filled with 200 mL of phosphate-buffered medium at a pH range of 7.7 to 10.25 to compare the effectiveness of the AOB and CDB for a combined nitrification and aerobic denitrification process in the presence and absence of F-CN. Additionally, pneumatic bioreactor systems (PBSs), with a maximum volume of 1.3 litre (1 litre working volume), were evaluated for nitrification/aerobic denitrification, using the AOB and CDB within the bioreactors. Similarly to BCs, the pH values that were assessed in the PBS were in the range pH 7.7 to 10.25. The pH range of cyanide-containing wastewaters is normally 9 to 11; therefore nitrification/aerobic denitrification under such conditions had to be evaluated. The concentration of ammonium was initially set at 1500 to 1600 mg  $\text{NH}_4^+$ /L for the BC/PBS under both non- and F-CN conditions. The PBS was constructed using polyvinyl chloride and had a maximum volume of 1.5 litres where the working volume was set at 1 litre. The internal diameter of the reactor was 100 mm and was covered with an airtight lid to prevent the escape of ammonium through stripping. The sampling port was located on the side of the reactor and the samples were taken using a 10 mL syringe. The schematic representation of the reactor is shown in Figure 3.2.

The robustness of the PBSs was evaluated using repeated cycles ( $n = 3$ ) in F-CN (8 mg F-CN/L) for a single unit nitrification/aerobic denitrification process, with ammonium (4500 mg  $\text{NH}_4^+$ -N/L) added to the process when the ammonium concentration reached  $\leq 25$  mg  $\text{NH}_4^+$ -N/L. The BCs and PBSs were inoculated with a pre-grown inoculum which was added at 10% (v/v) of the total operational volume of the bioreactors for each of the experiments. The BCs were incubated at 30 °C at a speed of 180 rpm, while the PBSs were operated at room temperature (24 to 27 °C) using an aeration rate of 2 L/min.



**Figure 3.2:** A schematic representation of the ammonium oxidation by the cyanide-degrading bacteria (CDB) in a pneumatic bioreactor. The process describes the connection between the cyanide-biodegradation process and the nitrification process by the same microbial consortia which are capable of degrading cyanide and ammonia oxidation. A (1, 2 & 3): Bioreactors in series where cyanide biodegradation takes place with subsequent production of ammonia; B: A combined nitrification and/or aerobic denitrification process where the AOB (Option 1) and CDB (Option 2) are utilised, with more emphasis on the CDB (D1); D2: Schematic diagram of the pneumatic bioreactor to which nitrification/aerobic denitrification by the CDB takes place

The schematic diagram represented in Figure 3.2 describes a proposed integrated process for the continuous biodegradation of free cyanide and subsequent nitrification and aerobic denitrification by CDB. In the first stage, free cyanide ( $\leq 500$  mg F-CN/L) is biodegraded in a three-stage bioprocess with subsequent formation of ammonia by the CDB. However, biodegradation efficiency during the process is rarely complete, hence, the residual cyanide runoff to the nitrification/aerobic denitrification stage (B). The process designed is such that traditional nitrifiers (AOB) are employed to accomplish the required nitrification and denitrification processes (option 1) when the residual cyanide concentration from the cyanide biodegradation stage is  $\leq 1$  mg F-CN/L. However, the AOB were unable to achieve significant nitrification and aerobic denitrification simultaneously compared with the CDB, hence, it was favourable to utilise the CDB as they were able to nitrify and aerobic denitrify (option 2) even in the presence of F-CN, which implied that the CDB consortia were appropriate for the nitrification-aerobic denitrification process in a PBS (E1), compared with the AOB, even at high ammonia and nitrate concentrations.

### **3.7.3 Effect of F-CN loading on nitrification and aerobic denitrification in a fed-batch PBS**

Nitrification and aerobic denitrification by AOB and CDB were investigated in PBSs with and without the presence of free cyanide (F-CN). The F-CN concentration was set at 2, 4 and 8 mg F-CN/L using KCN in a phosphate buffered media (pH 10.25), with the supply being intermittent and maintained at the required concentration. The  $\text{NH}_4^+/\text{NO}_3^-$  removal using AOB was compared with that of CDB in the presence of F-CN to assess the impact of cyanide on nitrification and aerobic denitrification in a single unit. The PBSs contained biofilm attachment carriers (Bioballs™, 20 cm<sup>3</sup>) and the bioreactor was operated in a fed-batch mode. The Bioballs™ were composed of acrylonitrile, butadiene and styrene (ABS). The media composition contained whey waste (40 g/L) in a phosphate- buffered solution.

## **3.8 Lab-scale packed bed system for the continuous degradation of free cyanide**

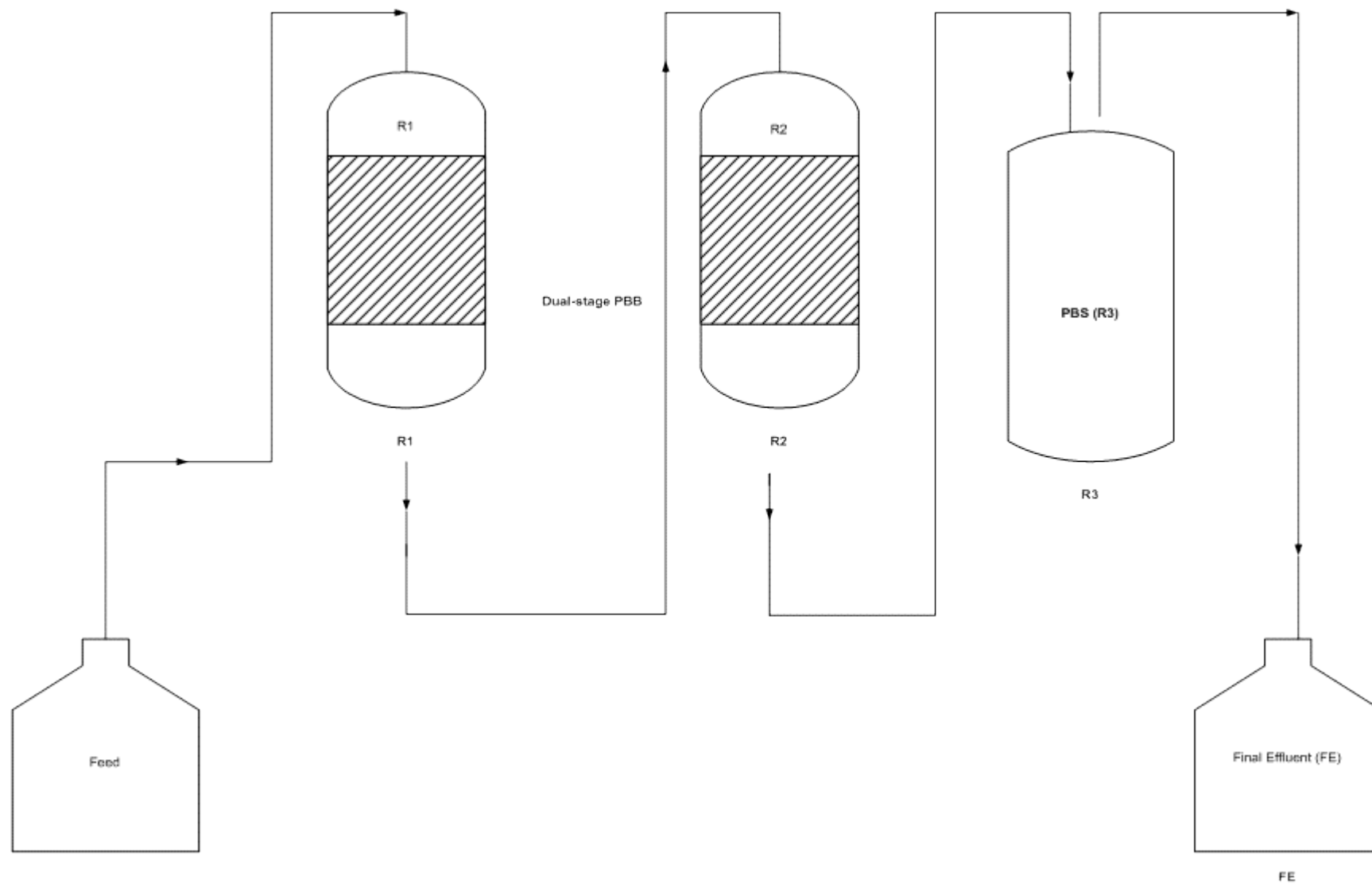
### **3.8.1 Column setup and system description**

The columns were constructed for the continuous biodegradation of cyanide in a packed system. A dual-stage packed-bed system, labelled Reactor 1 and 2 (R1 & R2 respectively), was designed where a pneumatic bioreactor acted as a third bioreactor (R3) where nitrification and aerobic denitrification took place. The total volume of the pneumatic bioreactor was 0.27 L and therefore was operating a residence time of 24 hours since the system flow was set at 0.26 L/day.

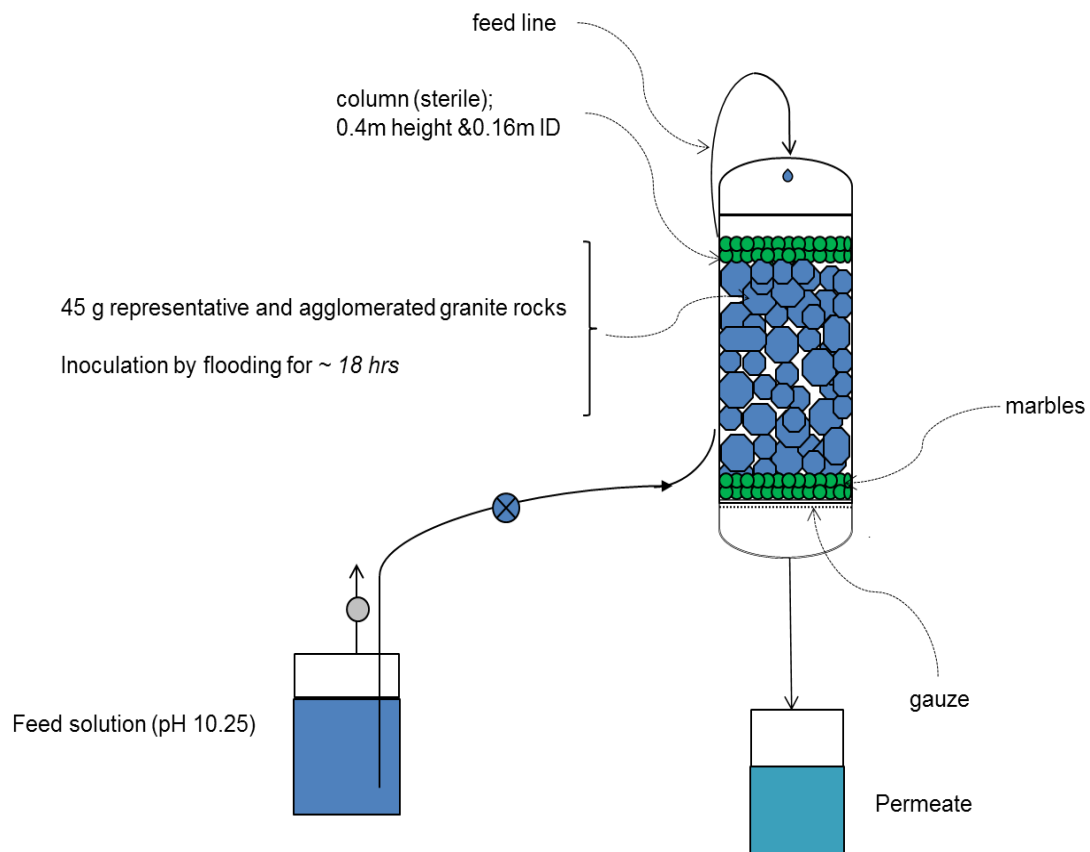
A dual-stage packed-bed bioreactor with a pneumatic bioreactor, serving as a third reactor, was constructed according to the schematic diagram shown in Figure 3.3 and 3.4. The dual-stage packed-bed reactors served as primary cyanide biodegradation stages, while the pneumatic bioreactor served primarily as a nitrification and subsequent aerobic denitrification reactor. All the materials used were sterilised at 121 °C for 20 minutes and the system was operated at the optimised temperature (33.60 °C), where the cyanide concentration was increased from 100 to 500 mg F-CN/L for a period of approximately 80 days. Each packed reactor had a collection bottle where the samples were taken. The bottles were airtight to prevent volatilisation of cyanide and the samples were taken using 20 mL BT syringes with a Luer-Lock tip.

Each column was packed with 44 g of agglomerated granite rocks (2 mm). Agglomeration was performed by mixing 10 mL of a phosphate-buffered solution (pH 7.00) with 44 g of granite rocks to ensure attachment of the smaller particles of the granite rocks to the larger rocks. Sterile marbles were placed on top and beneath the agglomerated granite rocks to assist with uniform liquid distribution. The columns were inoculated by aseptic flooding, where the microbial mixture was aseptically poured over the packed granite rocks and the flooded columns were left for 18 hours to allow the microorganisms to attach to the rocks. The solution was slowly drained before commencing with irrigation.

The feed solution was trickled on top of the granite rocks at a total flow rate of 0.26 L/day using the Watson Marlow 520S peristaltic pump. The system was not aerated and therefore the microbial species utilised the dissolved oxygen in water to fulfil their oxygen requirements.



**Figure 3.3:** A schematic diagram dual stage packed bed bioreactor (PBB) with a pneumatic bioreactor system (PBB). Reactor 1 (R1) and 2 (R2) served as cyanide biodegradation reactors while reactor 3 (R3) served as a nitrification/aerobic denitrification reactor.



**Figure 3.4:** A schematic representation of single packed bed bioreactor.

### 3.8.2 Biofilm detachment procedure from the packed-bed bioreactors

To assess microbial growth as the F-CN concentration increased, a modified detachment procedure was used to detach microbial cells from the granite (Govender *et al.*, 2013). A sterile phosphate buffer (pH 7.0) was used instead of the autotrophic basal salt (ABS) for detachment. The method differentiates between the interstitial, weakly-attached and strongly-attached cells. Phosphate buffer (pH 7.0) solution was used to detach the cells from the rocks. A granite sample weighing 2 g was placed into a sterile 100 mL conical flask and treated with 3 washing steps. Each washing step involved the addition of 2 mL of the phosphate buffer solution followed by vortexing for 2 minutes (except for the first step where the flask was swirled smoothly), followed by centrifugation at a relative centrifugal force of 800 g for 1 minute, decanting the supernatant and discarding the settled residue. Tween 20 was diluted in the detachment medium to achieve a concentration of 0.4 % v/v which was used in the last step to detach the strongly attached microorganisms. The centrifugation was done using a HERMLE Z 233 M-2 centrifuge (Lasec, South Africa). The solution from step 1 was regarded as the interstitial phase; the solution from step 2 was regarded as the weakly attached cells and the solution from the last step was regarded as the strongly attached cells (Govender *et al.*, 2013). A cell count was done on the solution from each of the washing steps of detachment.

### 3.9 Statistical analysis

The experimental error was calculated as the standard error of mean using the standard deviation obtained from the multiple sets of data ( $n = 3$ ). Equation 3.4 demonstrates the formula that was used to calculate the SEM.

$$\frac{\text{Standard deviation}}{\sqrt{\text{Number of samples tested}}} \quad (3.4)$$

### 3.10 Analytical methods

Before analysis, the samples were obtained from the shake flasks and centrifuged at 14 500 rpm for 5 minutes, followed by filtration through a 0.22 $\mu$ m filter, and thereafter analysed using the methods described below.

#### 3.10.1 pH measurement

The pH was measured using a Crison Basic20 pH meter. The electrode was calibrated daily using Crison buffer solutions (pH 4.00, 7.00 and 9.00).

#### 3.10.2 Spectrophotometric and photometric tests

The total sugar concentration was measured using the 3,5-dinitrosalicylic acid method (Miller, 1959). Merck ammonium ( $\text{NH}_4^+$ ) (00683), cyanide ( $\text{CN}^-$ ) (09701) and nitrate (14773) test kits were used to quantify the concentration of ammonium, free cyanide and nitrates in a Merck Spectroquant Nova 60 instrument. Briefly, the cyanide test kit works on the on the reaction of cyanide with chloramine-T and pyridine-barbituric acid (Lambert *et al.*, 1975). The ammonia test kit works on the Berthelot reaction between ammonia, chlorine and phenolic compounds to form indophenol dyes (Patton & Crouch, 1977). The nitrate test kit makes use of concentrated sulphuric acid in the presence of a benzoic acid derivative.

#### 3.10.3 Quantification of microbial species

Microorganisms were counted using two methods: the plating method and direct cell counting. In the plating method, nutrient agar was prepared according to the manufacturer's instructions (Merck, South Africa). After cooling the agar to about 50 °C, cyanide was added to accomplish a concentration of 100 mg F-CN/L and the agar was left to solidify. Appropriate sample dilutions were prepared in a phosphate- buffered medium (pH 7.0) and a spread plating technique was applied. The plates were stored in a 37 °C incubator for 48 hours.

Viable cell counts from the detachment procedure were counted directly using the THOMA Helber counting chamber under a Nikon Eclipse E2000 phase contrast microscope at 100X



magnification. The cell concentration (cells/mL) was determined from the cell counts by multiplying the number of cells counted, with a value of 312 500 supplied by the manufacturer (see Appendix A2). Samples with a high cell concentration were diluted to count between 30 and 300 cells, to minimise the counting error. This method has a detection limit of  $3 \times 10^5$  cells/mL with an error < 25%.

### **3.11 Physiological tests to study microbial species**

#### **3.11.1 Gram staining and endospore staining**

A smear was prepared by transferring a loopful of cells into a clean glass slide followed by heating fixing in a Bunsen burner. Pure cyanide tolerant bacterial cultures were identified using the Gram and endospore staining techniques.

#### **3.11.2 Starch hydrolysis**

Organisms grown from an overnight culture were streak plated on a starch agar plate and incubated at 37°C for 24 hours. Iodine solution was added on the overnight plates to assess whether the organisms hydrolysed the starch. If starch has been hydrolysed, a clear zone around the bacterial growth area forms. If starch has not been hydrolysed, the agar will remain a dark brown or blue/black colour.

#### **3.11.3 Oxidase activity**

Microbial cultures were grown in 4.5 mL of nutrient broth for 24 hours and thereafter in 0.2 mL of 1%  $\alpha$ -naphthol and 0.3 mL of 1% *p*-aminodimethylalanine oxalate, and colour change was observed. Microorganisms are oxidase positive when the colour changes to blue in 15 to 30 seconds. A purple colour suggests a delay in oxidase positive organisms and no colour change suggests that the organisms are oxidase negative.

### **3.12 Growth rates and reaction rate kinetics**

Growth rates and doubling time of the mixed cultures were calculated using Equation 3.5 and 3.6. In the growth rate calculation, it was assumed that:

- the growth of microorganisms on the granite rocks was a batch type of growth;
- the growth of the microorganisms followed first-order kinetics.

Therefore, the specific growth rate ( $\mu$ ) and its corresponding doubling time were calculated using the following equations (Doran, 1995):

$$\mu = \frac{(\ln x_2 - \ln x_1)}{t_2 - t_1} \quad (3.5)$$

$$t_d = \frac{\ln 2}{\mu} \quad (3.6)$$

where  $\mu$  is the specific growth rate constant ( $\text{h}^{-1}$ )

$x_1$  and  $x_2$  are the cell concentration at  $t_1$  and  $t_2$ , respectively

$t_d$  is the doubling time (h) i.e. when  $\frac{x_2}{x_1} = 2$

Reaction rate constant was calculated using the first-order kinetics as described in Equation 3.7.

$$[C] = [C]_0 e^{-kt} \quad (3.7)$$

where  $[C]$  is the final concentration (mg/L),  $[C]_0$  is the initial concentration (mg/L),  $k$  is the reaction rate constant ( $\text{s}^{-1}$ ) and  $t$  is time (s). Equation 3.7 was used to quantify nitrification and denitrification rates

### 3.13 DNA extraction, polymerase chain reaction (PCR) and 16S rDNA gene sequencing

Thirteen representative bacterial species from nutrient agar plates were isolated and single colonies were inoculated in 1ml nutrient broth and incubated at 30°C. DNA was extracted from the overnight cultures using the ZR Fungal/Bacterial DNA Kit (Zymo Research, California, USA). The presence of genomic DNA was visualised using 1% molecular-grade agarose gel containing 0.5 $\mu\text{g}/\text{ml}$  ethidium bromide (EtBr), and by using 1X Tris-acetate-ethylenediamine tetraacetic acid electrophoresis buffer at 100V for 1 hour.

PCR was performed using a GeneAmp PCR 9700 System (AppliedBiosystems, USA). Amplification of the target DNA by PCR was performed in a total reaction volume of 10 $\mu\text{l}$  containing 0.5 $\mu\text{l}$  ( $\pm 50 \text{ ng}/\mu\text{l}$ ) of the purified genomic DNA, 50mM of the forward and reverse primers and 5 $\mu\text{l}$  of a 2X KapaTaq Readymix solution (KapaBiosystems, South Africa). Bacterial specific primers used were the forward 8F primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 1492R 5'-GGTTACCTGTTACGACTT-3' (Turner *et al.*, 1999). The amplification process included an initial denaturing step at 94°C for 10 min, followed by 36 cycles of 94°C for 30s, 55°C for 30s and 72°C for 1 min. The reaction was completed with a final extension period of 7 min at 72°C followed by cooling and storage at 4°C. Ten microlitres of the PCR amplicons were electrophoretically analysed on a 1% molecular grade agarose gel that was stained with ethidium bromide, using 1X TAE electrophoresis buffer at 100V for 1 h, to determine whether the amplification was successful. The PCR amplicons were run on an ABI 3010xl Genetic

Analyzer. The sequences were blasted against the NCBI GenBank database and phylogenetic trees were generated using ClustalX for the alignment and PAUP for the analysis. Distance analysis using the neighbour joining algorithm and the strength of the branches was calculated with 1000 bootstrap repetitions.

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# **CHAPTER 4**

## **RESULTS AND DISCUSSION**

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## CHAPTER 4

### RESULTS AND DISCUSSION

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#### 4.1 Isolation, identification and cyanide biodegradation ability of the isolates

##### 4.1.1 Introduction

Bacterial growth proved to be more rapid compared to the growth of fungal and algal species. Therefore, it is suitable to isolate such species in cyanide-containing wastewater, as these organisms will degrade the cyanide more efficiently. Furthermore, a high cyanide tolerance of these organisms is imperative, so that they are able to degrade very high concentrations of cyanide.

Cyanide-tolerant bacteria were isolated from electroplating wastewater by suppressing the growth of fungal species. The identification of these species is important and their cyanide degradability needs to be evaluated.

##### 4.1.2 Aims and objectives

The aims and objectives were to:

- isolate bacterial species from electroplating wastewater;
- identify the isolated bacterial species using physiological- and molecular biology-based methods;
- assess the ability of the isolates to degrade F-CN in nutrient broth; and
- assess agricultural residues as potential supplements to support the microbial population in the degradation of F-CN.

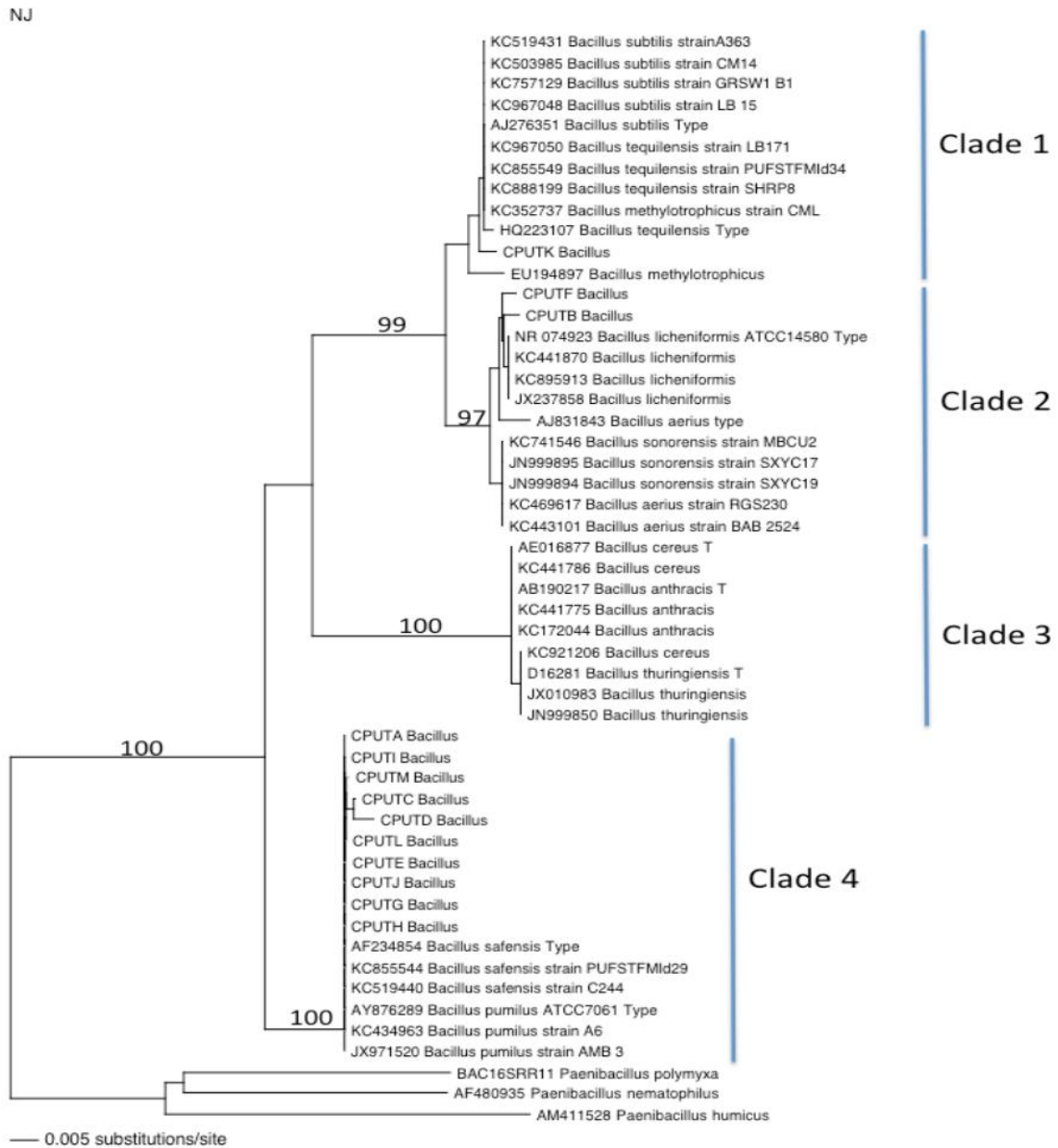
##### 4.1.3 Results and discussion

###### 4.1.3.1 Microbial isolation and identification

All the bacterial isolates (labelled CPUT A to CPUT M) were slender, aerobic, oxidase positive, gram positive and endospore-forming rods. The spores were located centrally in all the isolates. All the isolates could not hydrolyse starch, which was indicative of organisms that belong to the *Bacillus* genus as described by Meyers *et al.* (1991). The phylogeny of the representative organisms in the GenBank was analysed using the neighbour-joining algorithm in ClusterX (Fig 4.1). The phylogenetic tree was constructed from the organisms that were a representative of the isolates.

The sequence of the bacterial species labelled CPUT-K was found to belong to the *Bacillus subtilis* group (Fig 4.1, Clade 1), while organisms labelled CPUT-F & B were found to belong to the *Bacillus licheniformis* group (Fig 4.1, Clade 2). The organisms labelled CPUT-A, C, D, E, G, H, I & J were identified as species belonging to the *Bacillus safensis/pumilus* group (Fig 1, Clade 4). Since the 16S rDNA gene sequence does not distinguish between species

belonging to the *Bacillus* genera, more especially to *Bacillus safensis* and *Bacillus pumilus* species (Wang *et al.*, 2007), it is advisable to use the gyrase gene (*gyrB* gene) for the identification of species belonging to the *Bacillus* genus, especially to the *Bacillus subtilis* group, as this is the most efficient method of distinguishing between *Bacillus* species (Wang *et al.*, 2007).



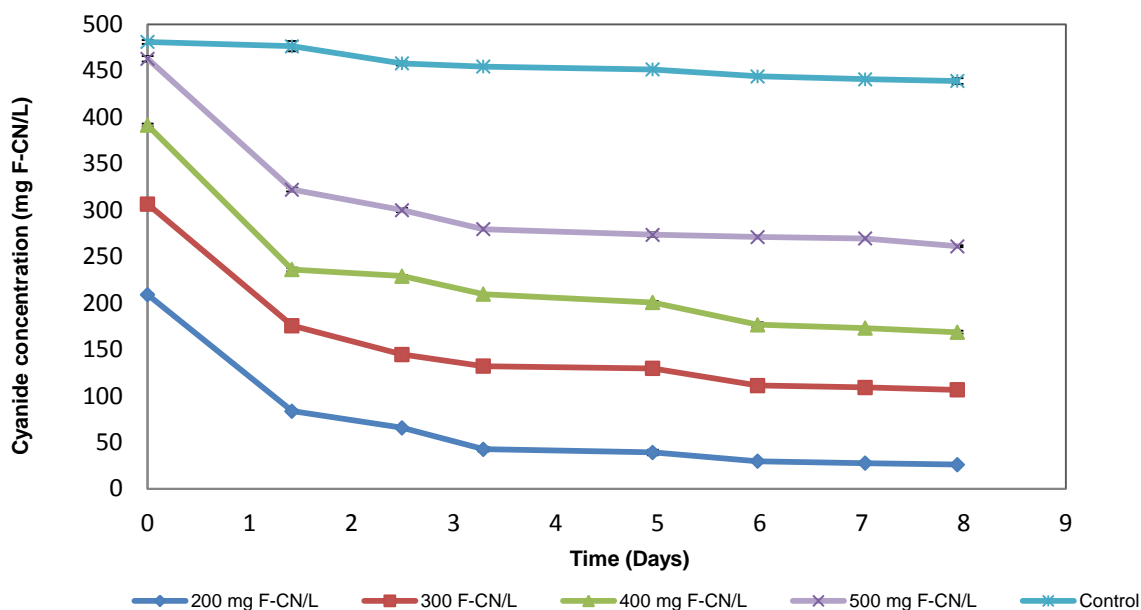
**Figure 4.1:** Phylogenetic neighbour-joining tree of the isolates based on the 16S rDNA gene sequence. A tree of 13 isolates was constructed using the Neighbour-joining algorithm of ClustalX.

However, the *gyrB* gene does not differentiate between *Bacillus safensis* and *Bacillus pumilus* as these organisms share 92% of the *gyrB* sequence similarity (Satomi *et al.*, 2006). Meyers *et al.* (1991) investigated the capability of the isolated cyanide-adapted *Bacillus*

*pumilus* to degrade free cyanide and observed that the organism was able to degrade 100 mg F-CN/L in 2 hours. Furthermore, *Bacillus pumilus* was found to possess the cyanide dihydratase enzyme which utilises the hydrolytic pathway for degradation of F-CN (Jandhyala *et al.*, 2005). There are currently no reports on the degradation of F-CN by *Bacillus subtilis* and *Bacillus licheniformis*. However, it has been reported that *Bacillus subtilis* is able to degrade organic cyanides (nitriles) through the action of nitrile hydratase (Zheng *et al.*, 2008). Additionally, it has been reported that the organism is able to express the nitrilase enzyme (Zheng *et al.*, 2008) and this enzyme has been observed to facilitate the degradation of F-CN (Gupta *et al.*, 2010).

#### 4.1.3.2 Biological degradation of cyanide

The mixed culture of the 13 isolates was prepared and assessed for the degradation of F-CN under alkaline conditions in nutrient broth. The residual F-CN in the medium containing 200 mg F-CN/L, 300 mg F-CN/L, 400 mg F-CN/L and 500 mg F-CN/L after 8 days was 26 mg F-CN/L, 106 mg F-CN/L, 170 mg F-CN/L and 261 mg F-CN/L respectively, with degradation efficiency of 87.6%, 65.4%, 57.0% and 43.6% respectively (Fig. 4.2). Using Equation 3.1, it was observed that the microorganisms were able to biodegrade 141 mg F-CN/L, 158 mg F-CN/L, 181 mg F-CN/L and 160 mg F-CN/L of F-CN from 200, 300, 400 and 500 mg F-CN/L of F-CN solutions, respectively, where it was observed that the spore-forming *Bacillus* species were able to withstand and degrade F-CN concentration in cultures containing F-CN concentration of up to 500 mg F-CN/L. It has been reported that the maximum concentration of F-CN that microorganisms can tolerate and be able to degrade is 200 mg F-CN/L (Kuyucak & Akcil, 2013). However, this study has proved that the microbial community employed in this system was able to degrade and tolerate cyanide concentrations of 500 mg F-CN/L. This is the first study to date to report on bacterial species that are able to tolerate and degrade F-CN efficiently in water containing such cyanide concentration. The ability of the white rot fungus, *Trametes versicolor*, to degrade F-CN at a maximum cyanide concentration of 400 mg F-CN/L, where the degradation efficiency was less than 30%, was evaluated elsewhere (Cabuk *et al.*, 2006). Furthermore, the immobilised cells of *Klebsiella oxytoca* were evaluated for the degradation of F-CN at a maximum concentration of 157 mg F-CN/L, where the microbe had obtained a degradation efficiency of 26% when cellulose acetate (CTA) was used (Chen *et al.*, 2008). Furthermore, in studies conducted by Cabuk *et al.* (2006) and Chen *et al.* (2008), aeration was used which results in the volatilisation of cyanide (Karavaiko *et al.*, 2000), making it difficult to conclude that degradation was due to biological degradation or volatilisation of cyanide.

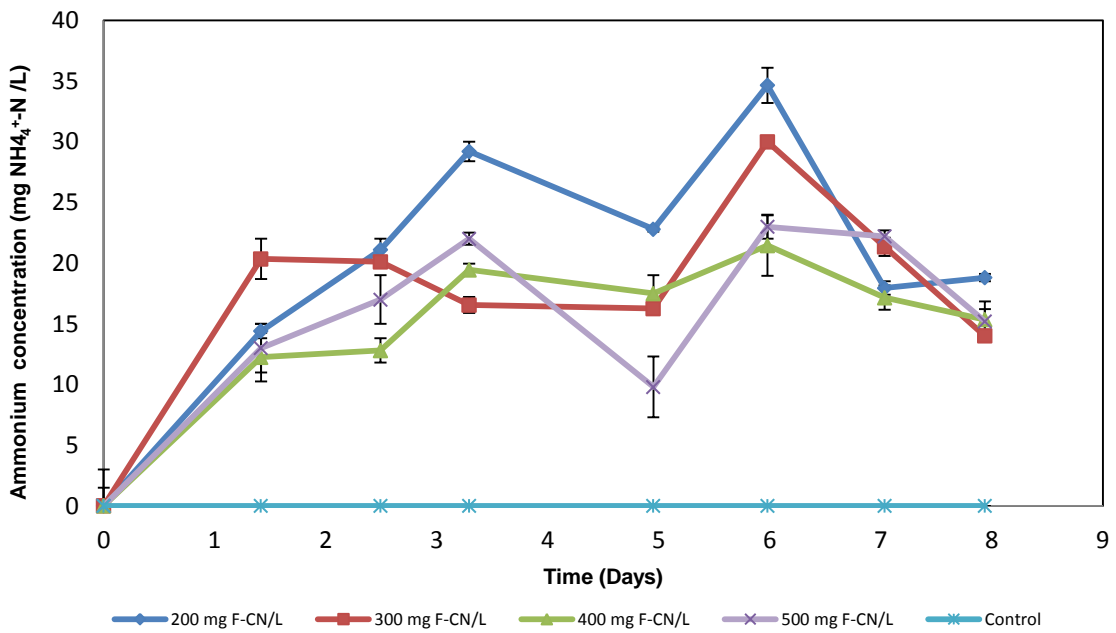


**Figure 4.2:** Cyanide biodegradation in nutrient broth by the bacterial consortium.

The residual ammonium concentration in nutrient broth fluctuated throughout the experiments, with the highest concentration being below 40 mg NH<sub>4</sub><sup>+</sup>/L (Fig. 4.3). The concentration of ammonium formed was dependent on the amount of cyanide that was biodegraded. The fluctuating concentration of ammonium was hypothesised to be largely dependent on ammonium utilisation by the microorganisms. It was, however, observed that the high ammonium concentration in the broth inhibited cyanide biodegradation, as the organisms prefer ammonium to cyanide as a nitrogen source.

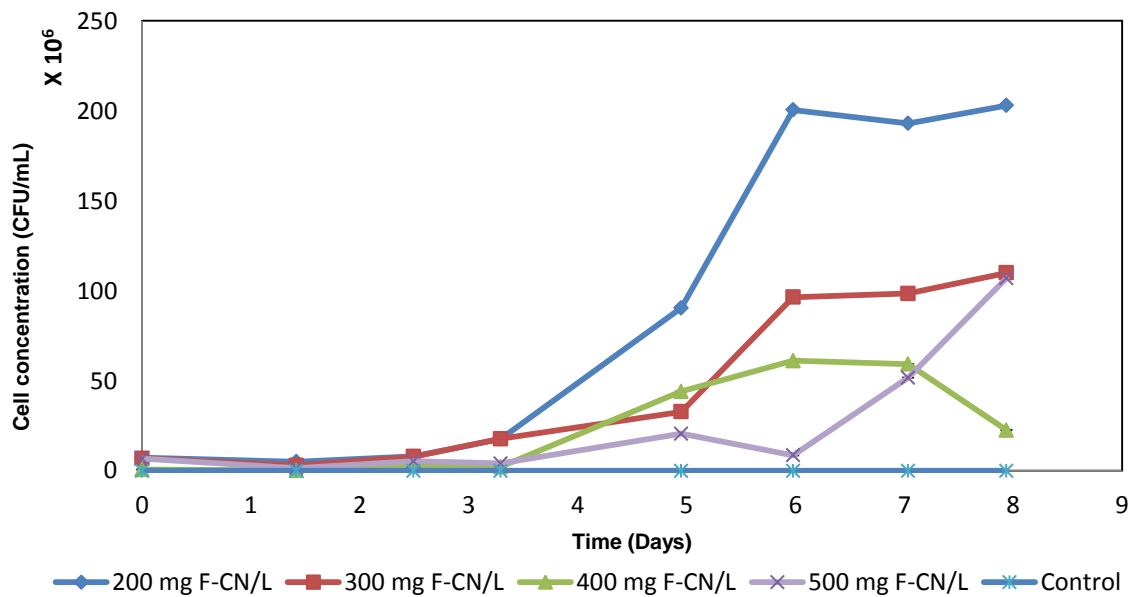
In this study, the degradation of cyanide was accompanied by increasing microbial growth, with cultures containing 200, 300, 400 and 500 mg F-CN/L showing maximum cell concentration of 2 x 10<sup>8</sup> CFU/mL, 1.1 x 10<sup>8</sup> CFU/mL, 6.1 x 10<sup>7</sup> CFU/mL and 1.07 x 10<sup>8</sup> CFU/mL respectively (Figure 4.4). Furthermore, the maximum specific growth rate of 1.46 d<sup>-1</sup>, 1.23 d<sup>-1</sup>, 1.23 d<sup>-1</sup> and 1.29 d<sup>-1</sup>, respectively, with their corresponding doubling time of 0.5 days, 0.56 days, 0.56 days and 0.54 days (Table 4.1) was achieved. The maximum growth rate when the cyanide concentration was set at 300 and 400 mg F-CN/L was the same, while the growth profiles were different. This might be due to the decline in microbial growth in the medium containing 400 mg F-CN/L after approximately 6 days. The decline in cell numbers in this medium might be due to depletion of nutrients in the media, thus leading to decreased cell numbers. In contrast, the media that contained 500 mg F-CN/L showed a prolonged lag phase of 6 days and thereafter the cell concentration increased exponentially to achieve a maximum growth rate of 1.29 d<sup>-1</sup>, with the corresponding doubling time of 0.54 days.





**Figure 4.3:** Residual ammonium concentration in the cultures during cyanide biodegradation in nutrient broth.

The microbial growth was highly dependent on the initial cyanide concentration in the cultures, with exponential growth being observed from days 3 to 6 in all cultures. Generally, the growth rate decreased as the cyanide concentration was increased in the cultures. As a result of this, the lag phase for cellular multiplication in the cultures increased (Fig. 4.4).

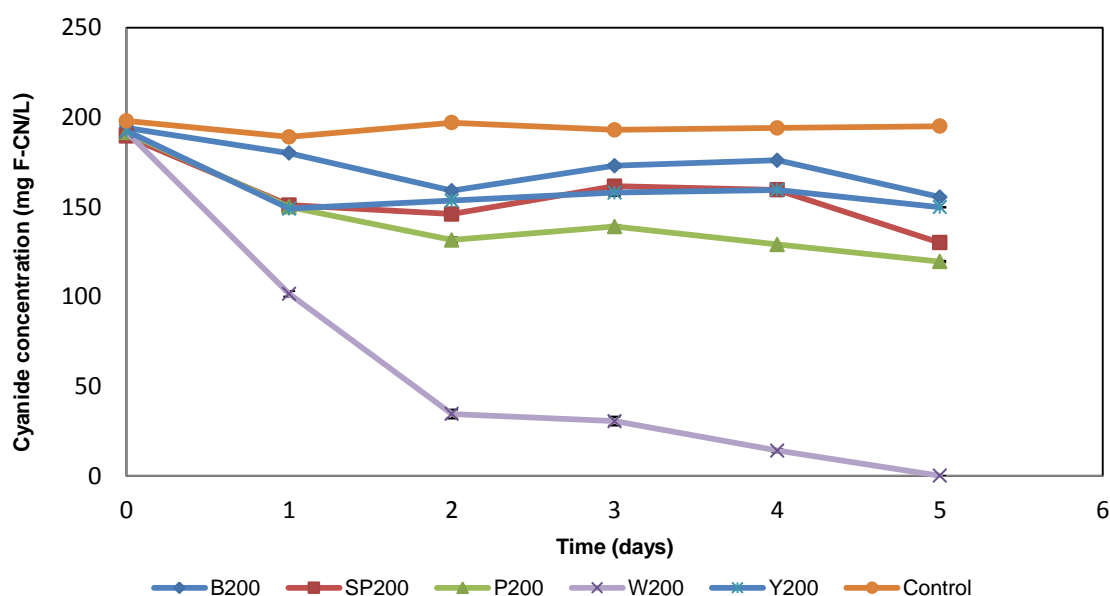


**Figure 4.4:** Microbial growth determined as cell concentration (CFU/mL) during cyanide biodegradation using nutrient broth.

**Table 4.1:** Maximum specific growth rates per cyanide concentration in nutrient broth

Growth media	$\mu_{\max}$ (day <sup>-1</sup> )	$t_d$ (days)
200 mg F-CN/L	1.46	0.50
300 mg F-CN/L	1.23	0.56
400 mg F-CN/L	1.23	0.56
500 mg F-CN/L	1.29	0.54

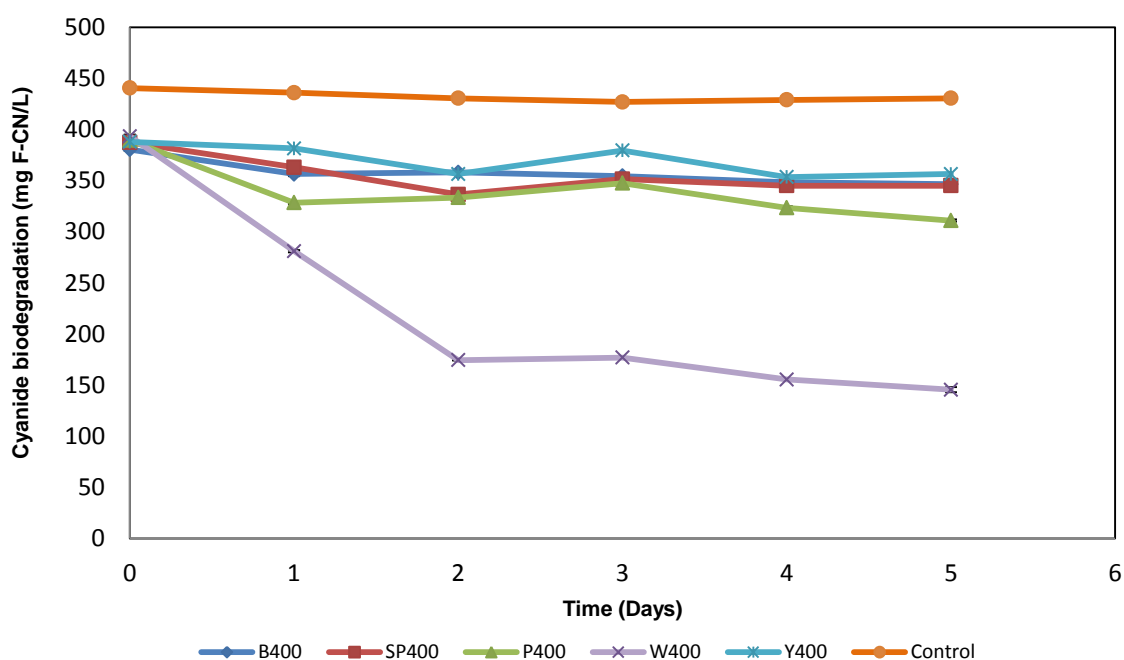
The bacterial isolates were able to degrade cyanide in media solely supplemented with agro-waste. However, the degradation efficiency was poor in media that were supplemented with *B. vulgaris*, *I. batatas*, *A. comosus* and spent brewer's yeast extracts at all the cyanide concentrations evaluated. The biodegraded cyanide using cultures with an initial cyanide concentration of 200 mg F-CN/L, for the *B. vulgaris*, *I. batatas*, *A. comosus* and spent brewer's yeast extracts was 27 mg F-CN/L, 47 mg F-CN/L, 60 mg F-CN/L and 32 mg F-CN/L, respectively, with the corresponding degradation efficiencies of 20%, 31.4%, 37.6% and 22.2%, respectively (Fig. 4.5).



**Figure 4.5:** Cyanide biodegradation in medium supplemented with agro-waste materials at a cyanide concentration of 200 mg F-CN/L. B200 – *B.vulgaris* at 200 mg F-CN/L, SP200 – *I.batatas* at 200 mg F-CN, P200 – *A.comosus* at 200 mg F-CN/L, W200 – Whey at 200 mg F-CN/L and Y200 – SBY at 200 mg F-CN/L.

Similarly, in cultures containing 400 mg F-CN/L, only 23, 23, 67 and 18 mg F-CN/L, respectively, were biodegraded with the corresponding degradation efficiencies of 9%, 11%,

20.5% and 8.1%. The degradation efficiency using these waste materials for microbial support was below 40%, with the medium that was supported with *A. comosus* extract showing the highest degradation efficiency of approximately 38%. The high degradation efficiencies experienced in the media that were supported by *A. comosus* extract are largely due to the high reducing sugar content in the extract (Ntwampe & Santos, 2013). However, when the cultures were supplemented with whey, the biodegradation efficiency was significantly increased. The residual cyanide in the media with 200 and 400 mg F-CN/L was 0.009 mg F-CN/L and 143 mg F-CN/L, respectively, after 5 days. This was translated into degradation efficiencies of 90% and 60% respectively (Fig. 4.6).

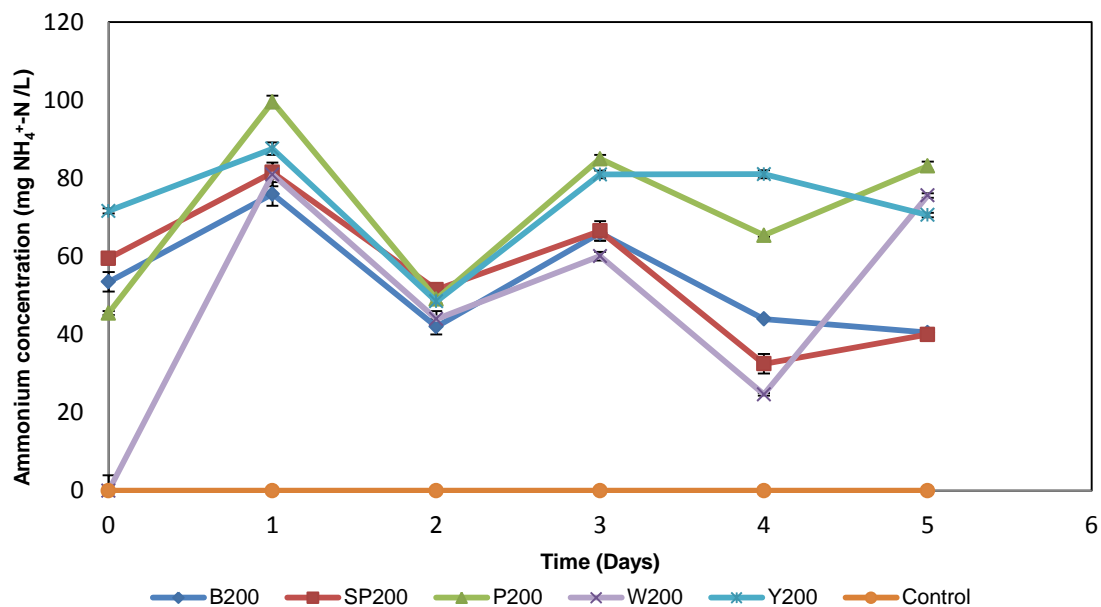


**Figure 4.6:** Cyanide biodegradation in medium supplemented with agro-waste materials at a cyanide concentration of 400 mg F-CN/L. B400 – *B.vulgaris* at 400 mg F-CN/L, SP400 – *I.batatas* at 400 mg F-CN, P400 – *A.comosus* at 400 mg F-CN/L, W400 – Whey at 400 mg F-CN/L and Y400 – SBY at 400 mg F-CN/L.

The biodegraded cyanide from a whey-supported media in cultures where the initial concentration was 200 and 400 mg F-CN/L was 179 and 239 mg F-CN/L, respectively (Figure 4.5 and 4.6). The degradation of cyanide in both the nutrient broth and whey-supplemented medium occurred within a period of 3 days. It is hypothesised that the degradation was due to the expression of cyanide-degrading enzymes in response to the high cyanide concentrations at the beginning of the experiments. Similarly, Akcil *et al.* (2003) evaluated the ability of the isolated *Pseudomonas sp.* to degrade high cyanide (weak acid dissociable [WAD]) concentrations (400 mg CN<sub>WAD</sub>/L) and observed that the species were

able to degrade cyanide efficiently, with degradation efficiency of approximately 90% occurring within a period of less than 5 days. However, the results presented did not contain any controls for comparison purposes, since cyanide is highly volatile. Furthermore, the use of shake flasks introduces aeration into the system, thus resulting in the chemical oxidation of cyanide (Karavaiko *et al.*, 2000). The initial ammonium concentration in cultures supplemented with agro-waste extracts in both the media containing 200 and 400 mg F-CN/L was high, except for the medium that was supplemented with whey (Fig. 4.7 and 4.8).

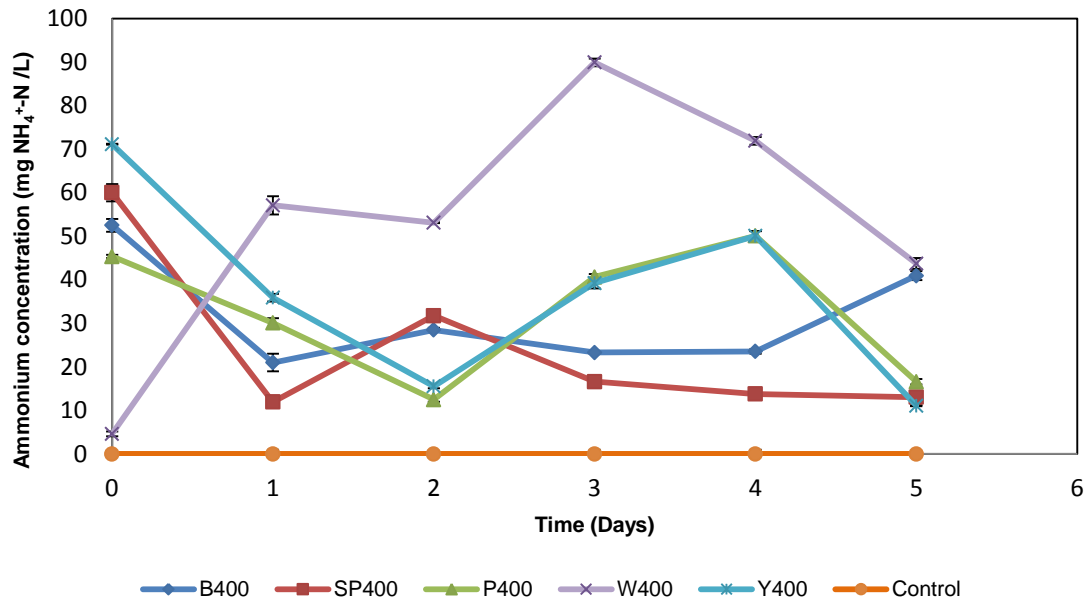
Since the microorganisms preferred the ammonium as a source of nitrogen to cyanide, this resulted in low cyanide degradation efficiency as the microbial species utilised the ammonium already present in the culture broth rather than the cyanide, with an exception to the medium that was supplemented with whey. The concentration of ammonium in all the flasks fluctuated with an observed maximum concentration of 76 mg NH<sub>4</sub><sup>+</sup>/L, 81.5 mg NH<sub>4</sub><sup>+</sup>/L, 99.6 mg NH<sub>4</sub><sup>+</sup>/L, 81 mg NH<sub>4</sub><sup>+</sup>/L and 87.6 NH<sub>4</sub><sup>+</sup>/L from a medium supplemented with *B. vulgaris* extract, *I. batatas* extract, *A. comosus* extract, whey and spent brewer's yeast extracts, respectively from a medium that contained 200 mg F-CN/L (Fig. 4.7).



**Figure 4.7:** Residual ammonium concentration in a medium supplemented with agro-waste at a cyanide concentration of 200 mg F-CN. B200 – *B.vulgaris* at 200 mg F-CN/L, SP200 – *I.batatas* at 200 mg F-CN, P200 – *A.comosus* at 200 mg F-CN/L, W200 – Whey at 200 mg F-CN/L and Y200 – SBY at 200 mg F-CN/L.

The maximum ammonium concentration in a medium that contained 400 mg F-CN/L from *B. vulgaris* extract, *I. batatas* extract, *A. comosus* extract, whey and spent brewer's yeast extracts was found to be 52.5 mg NH<sub>4</sub><sup>+</sup>-N/L, 60 mg NH<sub>4</sub><sup>+</sup>-N/L, 45.4 mg NH<sub>4</sub><sup>+</sup>-N/L, 89.9 mg

$\text{NH}_4^+\text{-N/L}$  and  $71.1 \text{ mg NH}_4^+\text{-N/L}$ , respectively (Fig. 4.8). The fluctuating ammonium concentration was due to formation and subsequent oxidation of the ammonium in the cultures. The degradation of cyanide and ammonium oxidation by the *Bacillus sp.* showed that these endospore-forming organisms are both cyanide degraders and nitrifiers, and therefore can be used in the reduction of residual ammonia.

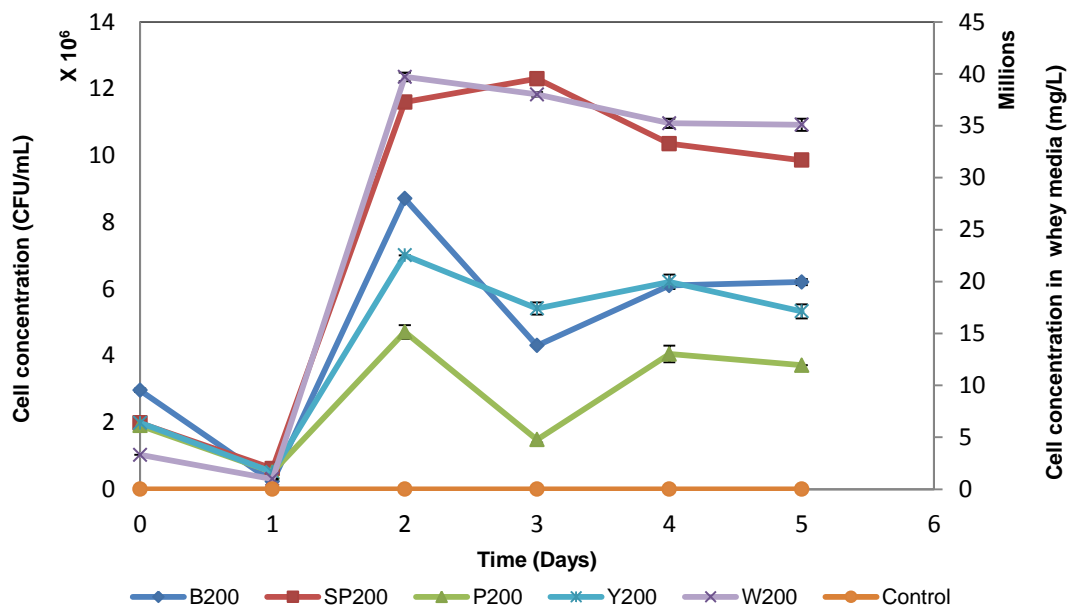


**Figure 4.8:** Residual ammonium concentration in a medium supplemented with agro-waste at a cyanide concentration of 400 mg F-CN. B400 – *B.vulgaris* at 400 mg F-CN/L, SP400 – *I.batatas* at 400 mg F-CN, P400 – *A.comosus* at 400 mg F-CN/L, W400 – Whey at 400 mg F-CN/L and Y400 – SBY at 400 mg F-CN/L.

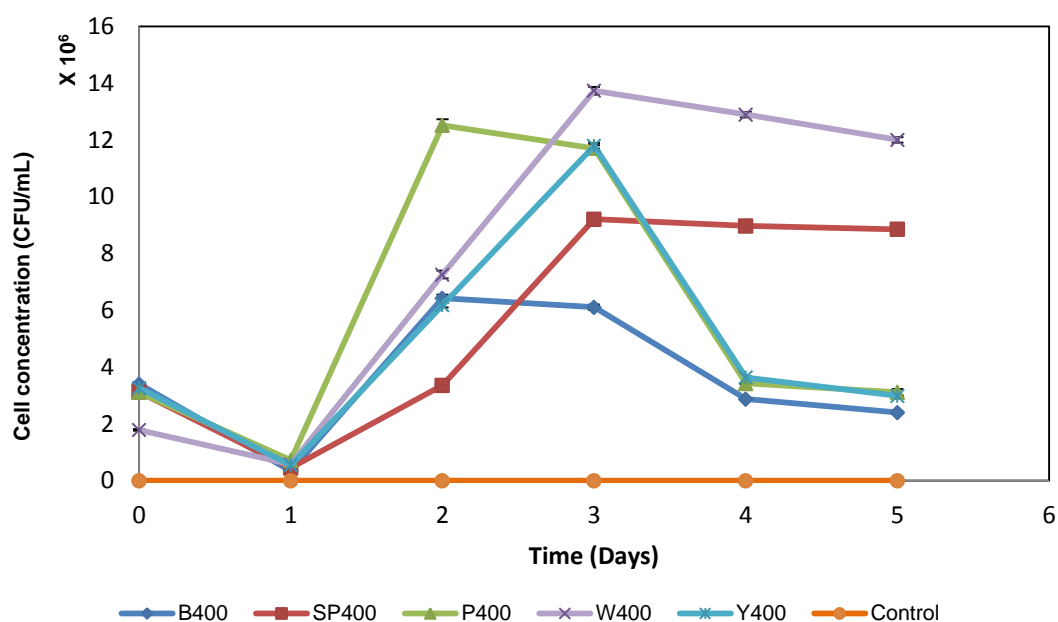
The maximum cell concentration in a medium supplemented with *B. vulgaris* extract, *I. batatas* extract, *A. comosus* extract, whey and spent brewer's yeast extracts at 200 mg CN/L was found to be  $8.71 \times 10^6$  CFU/mL,  $1.23 \times 10^7$  CFU/mL,  $1.25 \times 10^7$  CFU/mL,  $3.93 \times 10^7$  CFU/mL and  $1.18 \times 10^7$  CFU/mL, respectively (Fig. 4.9), while the maximum cell concentration at 400 mg CN/L was  $6.42 \times 10^6$  CFU/mL,  $9.21 \times 10^6$  CFU/mL,  $4.71 \times 10^6$  CFU/mL,  $1.36 \times 10^7$  CFU/mL and  $7.01 \times 10^6$  CFU/mL, respectively (Fig. 4.10). The observed maximum growth rate in both culture media containing 200 and 400 mg CN/L was  $3.64 \text{ d}^{-1}$ ,  $2.95 \text{ d}^{-1}$ ,  $2.18 \text{ d}^{-1}$ ,  $3.71 \text{ d}^{-1}$  and  $2.62 \text{ d}^{-1}$ , and  $2.92 \text{ d}^{-1}$ ,  $1.93 \text{ d}^{-1}$ ,  $3.40 \text{ d}^{-1}$ ,  $2.54 \text{ d}^{-1}$ ,  $2.54 \text{ d}^{-1}$  and  $3.07 \text{ d}^{-1}$ , respectively (Table 4.2).

The cell growth in a whey supplemented media containing 400 mg F-CN/L was similar to cell growth in the medium that contained less cyanide concentration, which suggested whey as a suitable substrate for the biodegradation of cyanide. The cell concentration observed was

similar to that obtained using nutrient broth, with a reduced lag phase period. Although the organisms were able to grow well on the agro-waste extracts, the media that were supported with *A. comosus* extract showed high cell concentrations, with a maximum cell concentration of  $1.25 \times 10^7$  CFU/mL and cyanide degradation efficiency of 37.6% (60 to 67 mg F-CN/L), which was comparable to the low F-CN degradation efficiencies that were observed in several studies (Chen *et al.*, 2008), where refined carbon sources were utilised for the degradation of cyanide. However, this was not observed for SBY, *B. vulgaris* and *I. batatas* supplemented cultures.



**Figure 4.9:** Microbial growth determined as cell concentration (CFU/mL) at a cyanide concentration of 200 mg F-CN/L. B200 – *B.vulgaris* at 200 mg F-CN/L, SP200 – *I.batatas* at 200 mg F-CN, P200 – *A.comosus* at 200 mg F-CN/L, W200 – Whey at 200 mg F-CN/L and Y200 – SBY at 200 mg F-CN/L.



**Figure 4.10:** Microbial growth determined as cell concentration (CFU/mL) at a cyanide concentration of 400 mg F-CN/L. B400 – *B.vulgaris* at 400 mg F-CN/L, SP400 – *I.batatas* at 400 mg F-CN, P400 – *A.comosus* at 400 mg F-CN/L, W400 – Whey at 400 mg F-CN/L and Y400 – SBY at 400 mg F-CN/L.

**Table 4.2:** Maximum specific growth rates per cyanide concentration in media supplemented with agricultural waste

Cyanide concentration (mg/L)	Agro-waste	$\mu_m$ (d <sup>-1</sup> )	$t_d$ (Days)
200	<i>B. vulgaris</i>	3.64	0.19
	<i>I. batatas</i>	2.95	0.24
	<i>A. comosus</i>	2.18	0.32
	Whey	3.71	0.19
	SBY	2.62	0.27
400	<i>B. vulgaris</i>	2.92	0.24
	<i>I. batatas</i>	1.93	0.36
	<i>A. comosus</i>	3.40	0.20
	Whey	2.54	0.27
	SBY	3.07	0.23

#### 4.1.4 Summary

All the isolated microorganisms from the cyanide containing wastewater belonged to the *Bacillus* genus. These microorganisms were found to be able to tolerate and degrade high concentrations of F-CN. This is the first report of bacterial species that can tolerate and degrade F-CN concentrations of 500 mg F-CN/L. Cyanide biodegradation was successful

even at elevated concentrations, with subsequent production of ammonium. However, the presence of ammonium was found to have an inhibitory effect on cyanide degradation as the microorganisms preferred utilising the ammonium over cyanide, thus reducing the efficacy of the cyanide biodegradation process. This phenomenon requires further studies on the application of these cyanide degrading microorganisms for the assimilation of ammonium that is generated through cyanide biodegradation. However, the formation of ammonium from cyanide degradation is of concern. It was observed that the cyanide-degrading organisms can alternate their metabolism depending on either the presence of cyanide or ammonium, thus utilising both substrates by activation/deactivation of metabolic pathway depending on the substrate that is present. The application of the same microbial species for both cyanide biodegradation and nitrification stages can add value to the designed process, increasing process efficiency and thus lowering operational costs of the process. The application of agro-waste, particularly whey-containing waste from the dairy industry, has been shown to successfully sustain microbial activity which in turn has resulted in an efficient process for the bioremediation of F-CN.

## **4.2 Nitrification and aerobic denitrification of high-strength ammonia-FCN containing wastewater**

### **4.2.1 Introduction**

The biodegradation of cyanide leads to the formation of ammonia and other by-products. However, ammonia is also an environmental hazard. After the biodegradation process, the wastewater containing elevated concentrations of ammonia can contaminate potable and surface waters. Furthermore, the biodegradation of cyanide is rarely complete; therefore, the discharged wastewater normally contains lower concentrations of cyanide compounds either as F-CN or in a complexed form. The microbial species normally employed in the nitrification and denitrification processes during municipal wastewater treatment, are susceptible to low levels of cyanide ( $\leq 1$  mg F-CN/L) (Kim *et al.*, 2011a, Kim *et al.*, 2011b). The presence of cyanide in these systems has been observed to result in the failure of the nitrification and denitrification process (Kim *et al.*, 2011a). This has negative implications for water re-use and discharge to surface waters.

Therefore, it is important to assess the capability of the cyanide-degrading bacteria to nitrify and denitrify the formed ammonia and nitrates in presence of F-CN.

### **4.2.2 Aims and objectives**

The aims and objectives were to:



- assess the ability of the isolates (CDB), in the form of a consortium, to carry out nitrification and aerobic denitrification with and without the presence of F-CN;
- compare the traditional nitrifying organisms, as ammonia-oxidising bacteria (AOB), with the isolated organisms, to carry out nitrification;
- evaluate the effect of F-CN loading on the nitrification and aerobic denitrification by the CDB and AOB; and
- assess the robustness of the CDB in nitrification and aerobic denitrification in a fed-batch system.

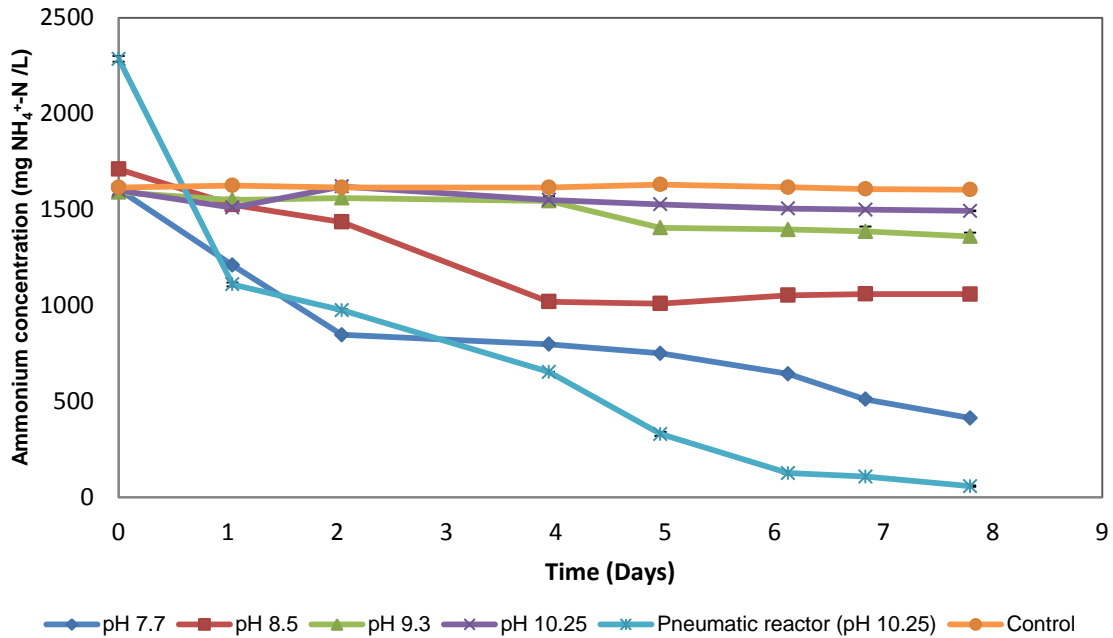
### 4.2.3 Results and discussion

#### 4.2.3.1 Nitrification by CDB and AOB

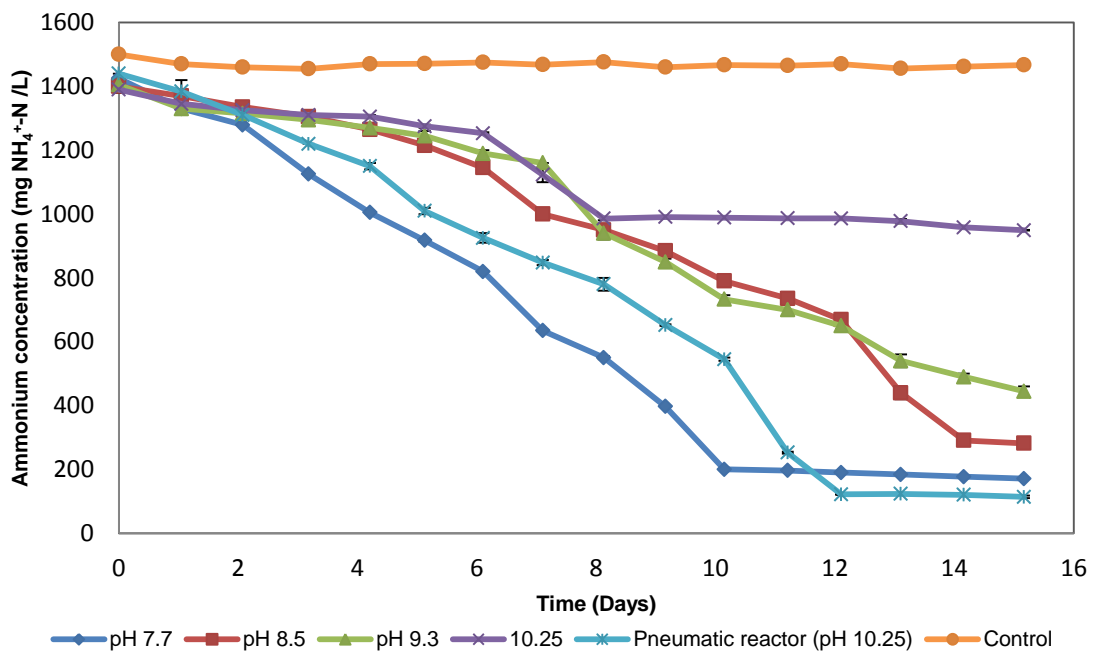
The ammonium utilisation by the CDB and AOB was evaluated in non-F-CN conditions. The concentration of ammonium was initially set at 1500 mg NH<sub>4</sub><sup>+</sup>-N/L for BCs and PBSs. The removal of ammonium was found to be pH dependent. At a lower pH of 7.7 and 8.5, the removal efficiency using CDB was found to be 74.16% and 38.13% in BCs, with minimal removal efficiency being achieved at a higher pH of 9.3 and 10.25, which achieved removal efficiencies of 14.47% and 6.69% (Fig. 4.11). Similarly, the ammonium removal by the AOB followed a similar trend to that of the CDB at lower pH in BCs (Fig.4.12). Although the AOB were able to utilise high ammonium concentrations efficiently, the period it took to oxidise the ammonium effectively was twice as long as that observed using CDB, achieving removal efficiencies of 88%, 79.86%, 68.33% and 31.73% for the cultures that were cultivated in a medium with an initial pH of 7.7, 8.5, 9.3 and 10.25, respectively, over a period of 15 days.

However, the removal efficiency of ammonium in the PBSs was high (97.31%), using CDB at pH 10.25, with 92% being achieved using AOB. The high removal efficiencies experienced in the PBS confirms the importance of oxygen mass transfer in the nitrification process. Ruiz *et al.* (2003) found that dissolved oxygen concentration of 1.7 mg/L was sufficient for successful AOB nitrification. Increased oxygen mass transfer improved nitrification significantly on comparison of BC and PBS cultures, which demonstrated the importance of oxygen mass transfer in heterotrophic and lithotrophic organisms that oxidise ammonia. The maximum ammonium removal was found at a pH of 7.7 in BCs, which is in agreement with the results obtained by Groeneweg *et al.* (1994). However, the removal of ammonium at highly alkaline pH environments (pH >10) has not been thoroughly explored and most of the microbial communities that are normally employed in nitrification are unable to oxidise ammonium efficiently at such pH values (Groeneweg *et al.*, 1994, Ruiz *et al.*, 2003, Strous *et al.*, 1997). This is a disadvantage when high-strength ammonium containing wastewater is remediated using AOB, which will thus reduce the efficiency of such a process. Therefore, the design and application of a CDB-facilitated alkaline nitrification process is important. In

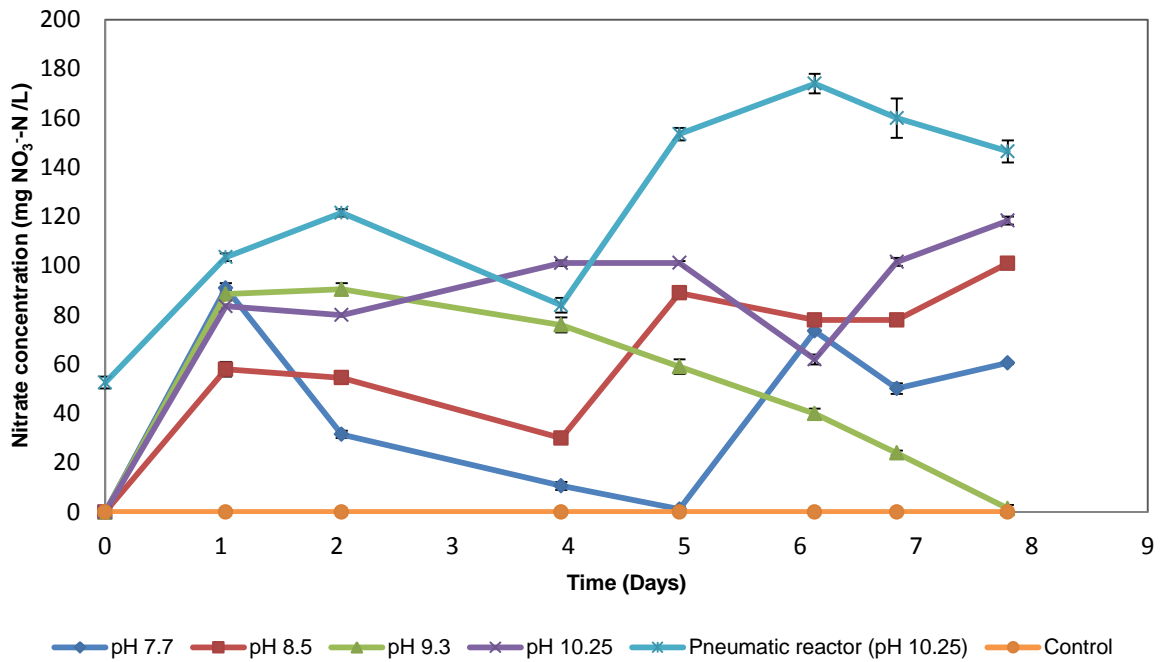
most cases, nitrification processes have been reported to be more efficient at pH ranges of 7.0 to 8.5 (Antoniou *et al.*, 1990, Groeneweg *et al.*, 1994, Koren *et al.*, 2000), while the information on nitrification under highly alkaline conditions is scarce. However, in this study, it was shown that *Bacillus* species are able to carry out nitrification in highly alkaline conditions.



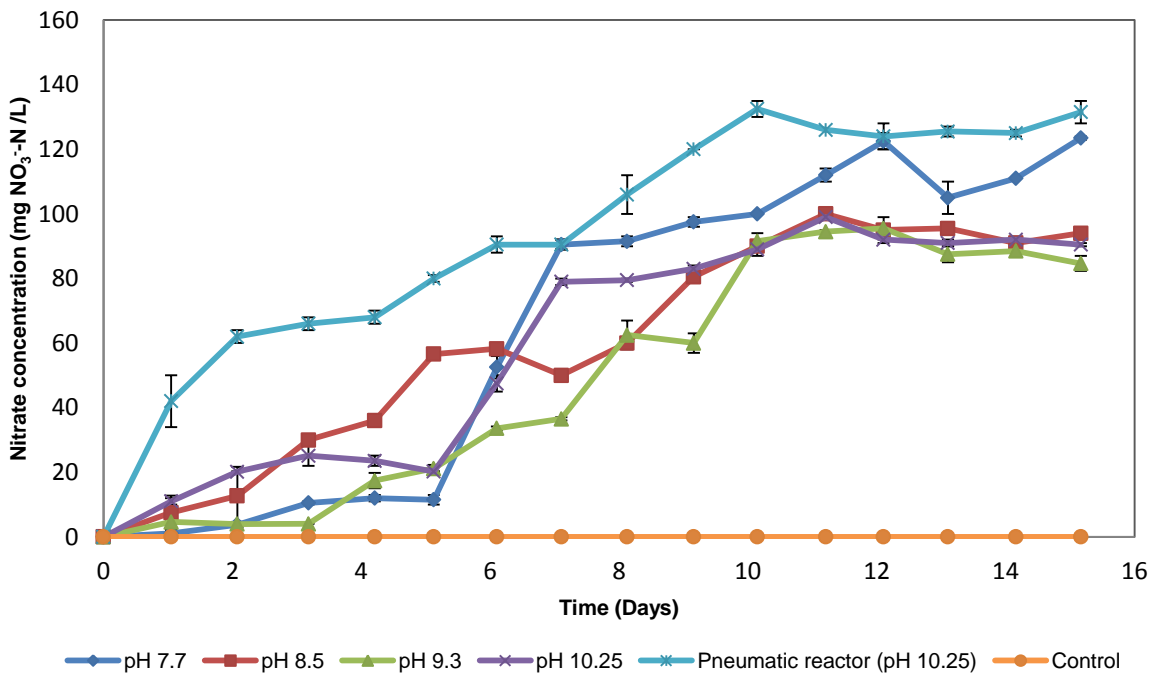
**Figure 4.11:** Ammonium removal by CDB in a medium solely supplemented with whey without F-CN



**Figure 4.12:** Ammonium removal by AOB in a medium solely supplemented with whey without F-CN



**Figure 4.13:** Nitrate profile from ammonium removal using CDB without F-CN



**Figure 4.14:** Nitrate profile from ammonium removal using AOB without F-CN

The assimilation of ammonium by the CDB resulted in the formation and accumulation of nitrates, where the nitrate concentration was at its highest (170 mg NO<sub>3</sub><sup>-</sup>-N/L) in the PBS at a pH suitable for nitrification (Fig. 4.13), with a similar trend being observed for the AOB-supported systems (Fig. 4.14). The CDB were found to be able to utilise the formed nitrates

at a lower pH value, while the cultures with an initial pH of 8.5 and 10.25 were unable to utilise the formed nitrates in BCs. However, aerobic denitrification in the culture with an initial pH of 9.3 was observed. In the PBS–CDB, an aerobic denitrification process was observed, thus implying that the CDB are able to denitrify under aerobic conditions. Nitrification and aerobic denitrification has been observed in previous studies (Chen *et al.*, 2012, Chen & Ni, 2012, Joo *et al.*, 2005, Zhang *et al.*, 2011). Microorganisms belonging to the *Bacillus* genus have also been found to be able to nitrify and denitrify aerobically. *Bacillus methylotrophicus* L7 oxidised ammonia effectively, achieving high nitrification rates with subsequent utilisation of the formed nitrates in an aerobic system (Zhang *et al.*, 2012), while Kim *et al.* (2005) observed that the *Bacillus* strains that were dominated by *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus cereus* were able to simultaneously nitrify and denitrify aerobically without the formation of nitrous oxide, which has been found to inhibit the nitrification process (Anthonisen *et al.*, 1976). However, there is a lack of information on the mechanism of aerobic denitrification and these studies report results for processes which are free of F-CN.

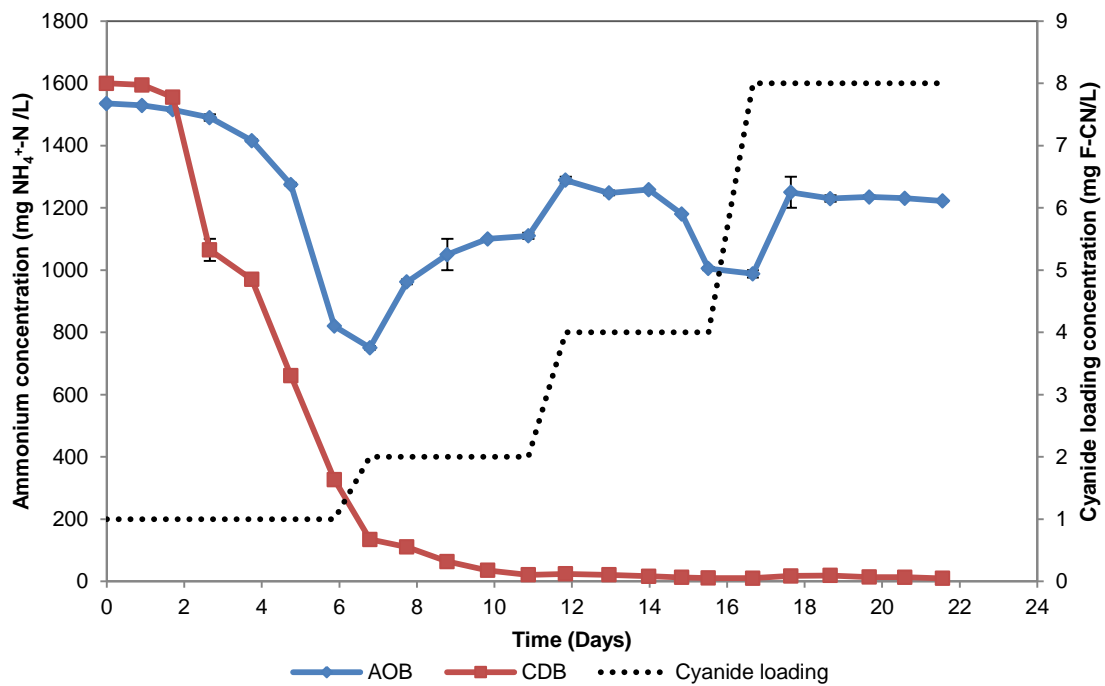
For the AOB experiments, the concentration of nitrates in the bioreactors (BC/PBS) accumulated throughout the experimental run without being utilised, with observed maximum nitrate concentration at pH 7.7 to 10.25 being 120 to 100 mg NO<sub>3</sub><sup>-</sup>-N/L in BCs, respectively, while in the PBS, the maximum observed was 130 mg NO<sub>3</sub><sup>-</sup>/L (Fig. 4.14).

#### **4.2.3.2 Effect of F-CN loading on combined nitrification and aerobic denitrification**

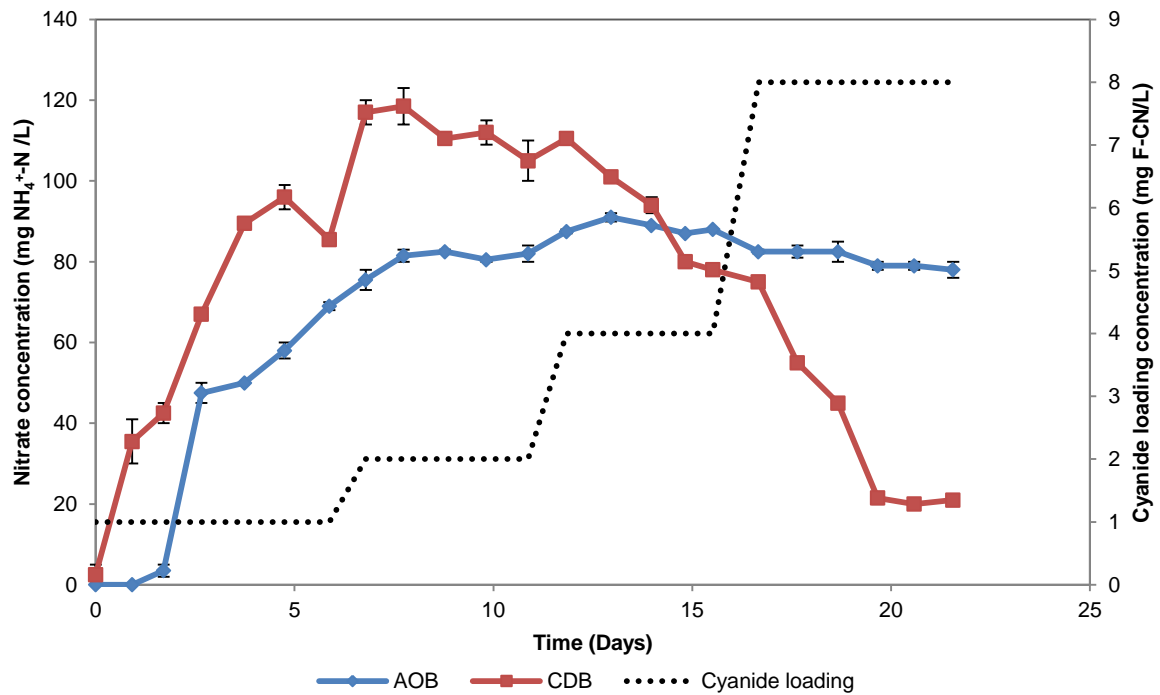
The effect of F-CN was evaluated in PBS by intermittently supplying, increasing and maintaining F-CN loading in the reactors at 1, 2, 4 and 8 mg F-CN/L, at five-day intervals (Fig. 4.15). Normally, cyanide biodegradation results in the formation of ammonium and bicarbonate. Additionally, during cyanide biodegradation, nitrification and denitrification systems are normally part of a series of process systems, with downstream systems being utilised for nitrification and denitrification using traditional nitrifying and denitrifying organisms. However, cyanide biodegradation is rarely complete, more especially during the cold season; therefore, the effect of F-CN on nitrification and denitrification using AOB and CDB, was evaluated to assess F-CN impact on these processes. Ammonium removal by CDB in F-CN was successful, achieving removal efficiency of 99%, irrespective of F-CN concentrations used during loading. This demonstrated that these microbial species can efficiently nitrify ammonium in the presence of cyanide.

In contrast, the AOB were not able to withstand the F-CN loading and as a result, the nitrification process failed when the system was loaded with ≥2 mg F-CN/L. The AOB were able to tolerate and oxidise the ammonium when the cyanide load was at 1 mg F-CN/L, with

removal efficiency being 51% after 7 days. It was also observed that the nitrification stagnated during the initial phase in AOB cultures owing to the presence of cyanide. Kim *et al.* (2008, 2011b) observed that the lag phase of nitrifying organisms increased when the cyanide concentration was set at 0.2 mg F-CN/L and nitrification was inhibited above 0.2 mg F-CN/L. It was, however, reported that the maximum concentration of F-CN to allow for nitrification to take place was 0.11 mg F-CN/L (Neufeld *et al.*, 1986); however, in this study, the species present within the sludge were able to tolerate and nitrify above the cyanide concentration threshold reported by Neufeld *et al.* (1986). Kim *et al.* (2008) investigated the effect of F-CN on nitrification by activated sludge obtained from a coking wastewater industry and found that a cyanide concentration above 1 mg F-CN/L inhibited the nitrification process; however, the activated sludge organisms were able to withstand 1 mg F-CN/L and oxidised ammonia, thus reaffirming the results obtained in this study.



**Figure 4.15:** Effect of cyanide loading on ammonium removal by AOB and CDB in a PBS in the presence of F-CN



**Figure 4.16:** Effect of cyanide loading on nitrate accumulation and/or removal by AOB and CDB

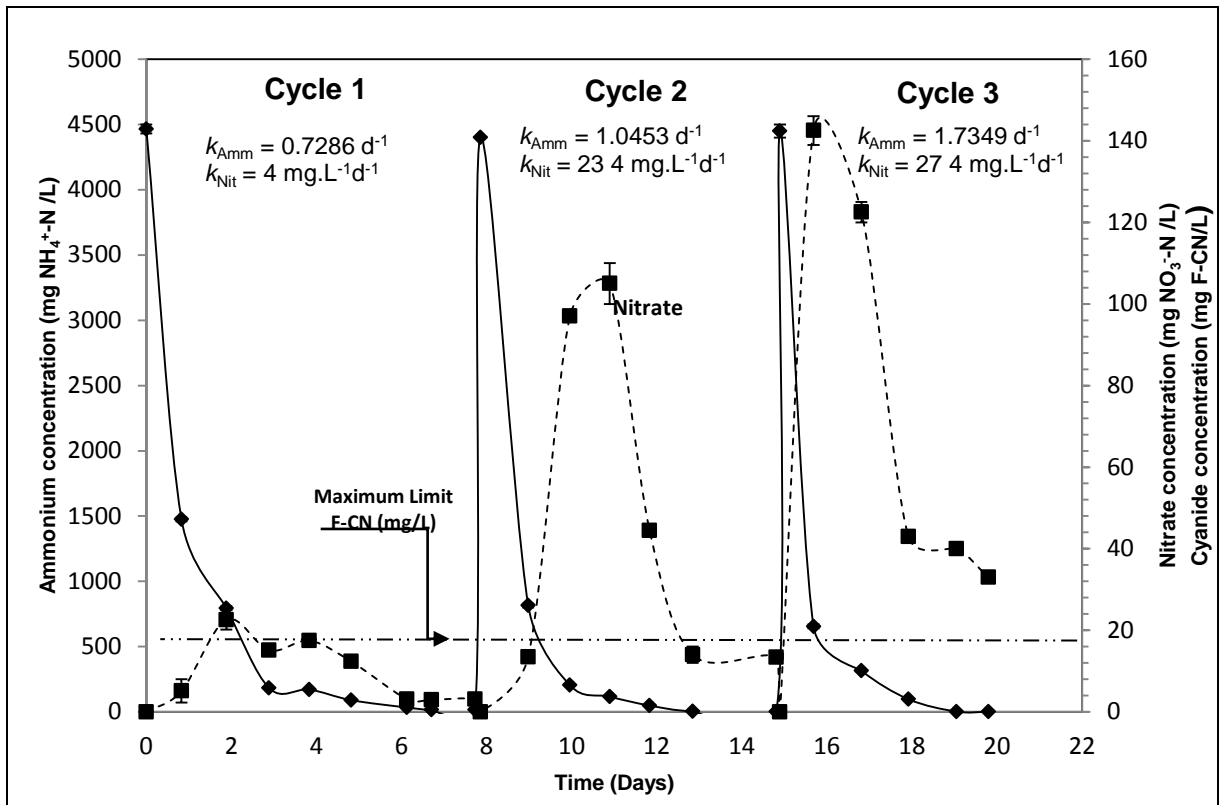
It was reported that species belonging to the *Nitrosomonas* and *Nitrosospira* were able to tolerate F-CN up to a concentration of 50 mg F-CN/L (Kim *et al.*, 2011a). However, in this study, it was observed that the AOB were not able to tolerate F-CN concentration above 2 mg F-CN/L when cyanide loading was increased. The high tolerance that has been reported in some studies, including that of Kim *et al.* (2011a), might be due to differences in the composition, strength and overall quality of the activated sludge used. It was observed that the ammonium accumulated in the bioreactors up to a concentration of 1100 mg NH<sub>4</sub><sup>+</sup>-N/L after 11 days; when the concentration of F-CN was increased to 4 mg F-CN/L on day 11 (after sampling), the accumulation of ammonium increased to approximately 1300 mg NH<sub>4</sub><sup>+</sup>-N/L, and thereafter, it slightly decreased to 976 mg NH<sub>4</sub><sup>+</sup>-N/L, before the cyanide load was increased to 8 mg F-CN/L. The AOB sparingly utilised the ammonium during days 4 to 5 at each F-CN loading stage, that is, after the initial F-CN was increased. This was also observed between days 14 to 17. Thereafter, the cyanide load was increased to 8 mg F-CN/L, which was subsequently followed by an increase in ammonia to approximately 1200 mg NH<sub>4</sub><sup>+</sup>-N/L.

The increase in ammonium concentration after cyanide loading was due to cell death or disruption owing to the toxicity of cyanide to the microbial species, which can result in cell disruptions, releasing ammonium-related compounds which lead to an increase in the ammonium concentration in the media. This phenomenon has been reported in previous

studies (Kim & Kim, 2003), where the authors observed excessive foaming and decreased microbial activity after cyanide loading. However, in this study, the observed increase in ammonium concentration was only attributed to cyanide conversion by surviving microorganisms during each cyanide loading stage, with minimal nitrification of the formed ammonium. This is because during days 3 to 6, some of the microorganisms constituting the AOB consortium were observed to tolerate a cyanide concentration of 1 mg F-CN/L during nitrification. One mechanism used by microorganisms in the presence of a toxicant, is to nullify its effects or convert it into a less harmful by-product or metabolically usable compound. For this study, increases in ammonium were observed when the cyanide load was increased, meaning that the F-CN was being converted into ammonium with minimal nitrification occurring at higher cyanide loads – particularly for AOB. However, for CDB in PBS, the cyanide load did not affect the nitrification process, with a rapid nitrification being observed between days 2 to 8. However, the concentration of nitrates in the reactor increased exponentially in both cultures (Fig. 4.16). The maximum nitrate concentration for the CDB and AOB was 123 and 90 mg NO<sub>3</sub><sup>-</sup>-N/L, respectively. It was observed that the AOB were not able to utilise the formed nitrates, while the CDB utilised the nitrates with the utilisation efficiency of 82% from day 8 to day 22.

#### **4.2.3.3 Combined nitrification and aerobic denitrification using fed-batch PBS-CDB in the presence of F-CN**

Ammonium removal was studied in a PBS operated in a fed-batch mode, with three successive cycles to assess the robustness of the CDB for combined nitrification and aerobic denitrification (Fig. 4.17). In the first, second and third cycle, the CDB removed ammonium and nitrates successfully, obtaining efficiencies of >99% for nitrification and 76% to 98% for aerobic denitrification, respectively. The removal of ammonium in the first, second and third cycle was for 8, 5 and 4 days respectively, where the nitrification rate constant (k) was 0.7286 d<sup>-1</sup>, 1.0453 d<sup>-1</sup> and 1.7349 d<sup>-1</sup>, with the removal rate of nitrates being 4 mg.L<sup>-1</sup>.d<sup>-1</sup>, 23 mg.L<sup>-1</sup>.d<sup>-1</sup> and 27 mg.L<sup>-1</sup>.d<sup>-1</sup>, respectively.



**Figure 4.17:** Combined single-step nitrification and aerobic denitrification in the presence of F-CN ammonium removal by CDB in fed-batch pneumatic bioreactor packed with microbial attachment surfaces

In this study, it was found that the reaction kinetics for the heterotrophic nitrification and aerobic denitrification follow a first-order kinetics, in contrast with the autotrophic nitrification process which has been reported to follow a zero-order kinetics (Wong-Chong & Loehr, 1975, Wong-Chong & Loehr, 1978). The increase in the reaction rate constants from cycle 1 to cycle 3 confirmed the robustness of the CDB species in removing ammonium and nitrates in the presence of F-CN. Additionally, the formed nitrates were utilised by the *Bacillus* consortium, especially under conditions where the ammonia was almost depleted in the wastewater, thus acting as aerobic denitrifying organisms, and thereby confirming the results reported by Yang *et al.* (2011) and Chen *et al.* (2012), in which *Bacillus subtilis* A1 and *Rhodococcus sp* CPZ24 utilised the nitrates when ammonium was depleted from the wastewater.

#### 4.2.4 Summary

The CDB were found to be able to utilise high concentrations of ammonium successfully, although the utilisation efficiency was dependent on the pH of the wastewater. High initial pH (10.25) inhibited nitrification in BCs, while the nitrification at lower pH values (7.7) was successful. However, the bacterial consortium was able to remove ammonium at high initial



pH value in the PBS, thus demonstrating the importance of oxygen mass transfer during the nitrification process. F-CN loading in the PBS did not affect the nitrification performance by the CDB, but inhibited the AOB at the cyanide-loading concentration greater than 2 mg F-CN/L. Furthermore, it was observed that cyanide loading resulted in reduced microbial activity when the AOB were used, thus resulting in an increase in ammonium concentrations in the wastewater. Additionally, it was observed that the CDB oxidised the ammonium successfully in fed-batch PBS, obtaining efficiencies beyond 99% in multiple cycles evaluated with subsequent nitrate utilisation, especially when the ammonium was depleted in the PBS. The *Bacillus* species were also observed to be capable of combined nitrification and aerobic denitrification, thus serving as cyanide degraders, nitrifying, and aerobic denitrifying organisms. Therefore, these microbial species can be employed successfully in the degradation of cyanide and subsequent utilisation of the formed ammonium and nitrates from cyanide biodegradation, as the traditional nitrifying organisms show susceptibility towards F-CN. Furthermore, the application of the PBS or highly aerated systems has been shown to accelerate the nitrification and aerobic denitrification, even when the initial pH of the wastewater is high, which is a typical characteristic of the wastewater containing cyanide.

### **4.3 Optimisation using response surface methodology**

#### **4.3.1 Introduction**

Optimisation of bioprocesses still remains one of the core elements in bioprocess engineering for increased productivity or efficiency. Independent variables can significantly influence process performance and therefore these variables need to be optimised so that the process performs at its maximum.

The effect of temperature, pH and substrate concentration, amongst others, needed to be evaluated and optimised using statistical methods such as response surface methodology.

#### **4.3.2 Aim and objective**

The aim and objective were to:

- Optimise the independent variables (pH, whey concentration and temperature), using response surface methodology for successful cyanide biodegradation.

#### **4.3.3 Results and discussion**

##### **4.3.3.1 Central composite experimental design**

The central composite design was employed to study the interactions among the independent variables that are presented in Table 3.2 and also determine their optimal levels. Table 4.3 shows the data emanating from the experiment of the effect of pH (A), whey

concentration (B) and temperature (C) on the biodegradation of cyanide. The experimental results were analysed through RSM to obtain an empirical model for the best response. The increased biodegradation of 213 mg F-CN/L observed in run 13 (Table 4.3), after a 96-hour incubation period, showed that higher substrate concentrations increase the rate at which organisms degrade pollutants, while in cultures with low substrate concentrations, the biodegradation decreases, probably due to depletion of the substrate in media to support microbial growth. The observed and predicted results based on the generated quadratic model (Eq. 3), is shown in Table 4.4.

**Table 4. 3:** Central composite design for 3 variables and the corresponding responses

Run	A	B	C	F-CN biodegradation (mg/L)
1	-1	-1	+1	60.5
2	-1	+1	+1	143
3	+1	-1	-1	86.5
4	+1	-1	+1	14
5	0	0	0	189
6	0	0	+ $\alpha$	68
7	+1	+1	-1	69.5
8	+ $\alpha$	0	0	50.499
9	-1	+1	-1	98
10	+1	+1	+1	89
11	0	0	- $\alpha$	95.5
12	0	- $\alpha$	0	54
13	0	+ $\alpha$	0	212.996
14	-1	-1	-1	94
15	- $\alpha$	0	0	184

**Table 4.4:** Observed and predicted responses obtained using CCD

Run no.	Observed (mg/L)	Predicted (mg/L)	Residual
1	60.5	64.54	-4.04
2	143	174.61	-31.61
3	86.5	74.27	12.23
4	14	2.67	11.33
5	189	189.78	-0.78
6	68	57.24	10.76
7	69.5	84.84	-15.34
8	50.5	59.08	-8.59

9	98	128.71	-30.71
10	89	98.49	-9.49
11	95.5	78.85	16.65
12	54	69.07	-15.07
13	213	170.51	42.48
14	94	103.89	-9.89
15	184	148	36

#### 4.3.3.2 Statistical model analysis and validation

The statistical model based on the sequential model sum of squares and lack-of-fit test explained the fitness of mean and reduced quadratic model for cyanide biodegradation (Table 4.5). A model (Eq. 4.2) was obtained that could relate to cyanide biodegradation quantified as measured output to the independent input variables. Analysis of variance (ANOVA) was used to assess the significance of each variable in the model. Multiple regression analysis was used to analyse the data and thus the polynomial equation derived from the regression analysis was:

$$Y = 189.78 - 26.44A + 30.16B - 6.43C - 30.49A^2 - 24.75B^2 - 43.04C^2 - 3.56AB - 8.06AC + 21.31BC \quad (4.1)$$

where A, B and C are coded values for pH, whey concentration and temperature, respectively. By considering the coefficients with significant effects ( $p < 0.05$ ) as shown in Table 4.5, Eq. 4.1 was reduced to:

$$Y = 189.78 - 26.44A + 30.16B - 30.49A^2 - 24.75B^2 - 43.04C^2 + 21.31BC \quad (4.2)$$

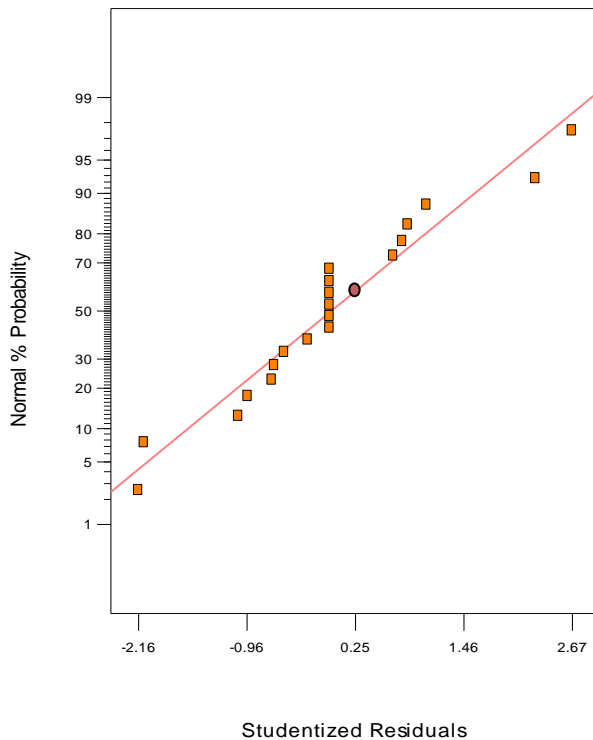
Using ANOVA, the adequacy of the model was assessed through the use of Fisher's statistical analysis and the results are shown in Table 4.5. The model  $F$  value of 11.75 implies that the model used was significant. In this case, the  $R^2$  (0.914) denotes a good correlation between the experimental and the predicted values. The  $P$  values obtained denote the significance of the coefficients in the model, highlighting interactions between the variables. The  $P$  values suggest that among the three variables studied, A (pH) and B (whey concentration) showed that they had a significant impact on the primary output – cyanide biodegradation. Adequate precision measures the signal to noise ratio with a desirable ratio of 4. The adequate precision ratio of 10.417 obtained in this study indicates an adequate signal. The coefficient of variance (CV) indicates the degree of precision with which the experiments are compared. The lower reliability of the experiment is normally denoted by a high CV value. The high CV value of 20.72 underscored the precision and reliability of the

model. The calculated squared correlation coefficient ( $R^2 = 0.914$ ) was good for the model. It is important to confirm the adequacy of the model to ensure that it gives a sufficient approximation of the actual test. The residuals from the least squares fit play an important role in judging model adequacy, and this can be done by constructing a normal probability of the plot of the residuals as shown in Figure 4.18. The normality assumption was satisfactory as the residual plot approximated along a straight line.

**Table 4.5:** Analysis of variance (ANOVA) for the quadratic model

Source	Sum of squares	DF	Mean square	F value	Prob > F
Model	68298.22	9	7588.69	11.75	0.0003
A	9543.65	1	9543.65	14.78	0.0032
B	12423.08	1	12423.08	19.24	0.0014
C	563.82	1	563.82	0.87	0.3721
A <sup>2</sup>	13398.15	1	13398.15	20.75	0.0011
B <sup>2</sup>	8825.13	1	8825.13	13.66	0.0041
C <sup>2</sup>	26698.45	1	26698.45	41.34	< 0.0001
AB	101.53	1	101.53	0.16	0.7001
AC	520.03	1	520.03	0.81	0.3906
BC	3633.78	1	3633.78	5.63	0.0391
Residual	6458.34	10	645.83	-	-
Lack of fit	6458.34	5	1291.67	-	-

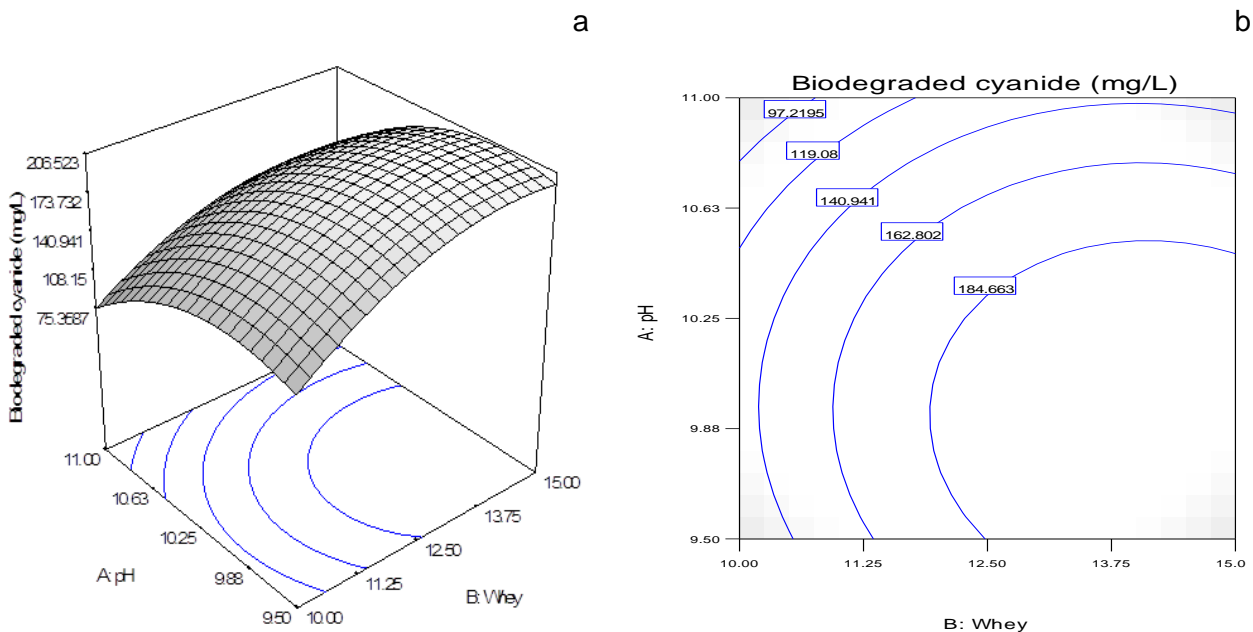
$R^2 = 0.914$

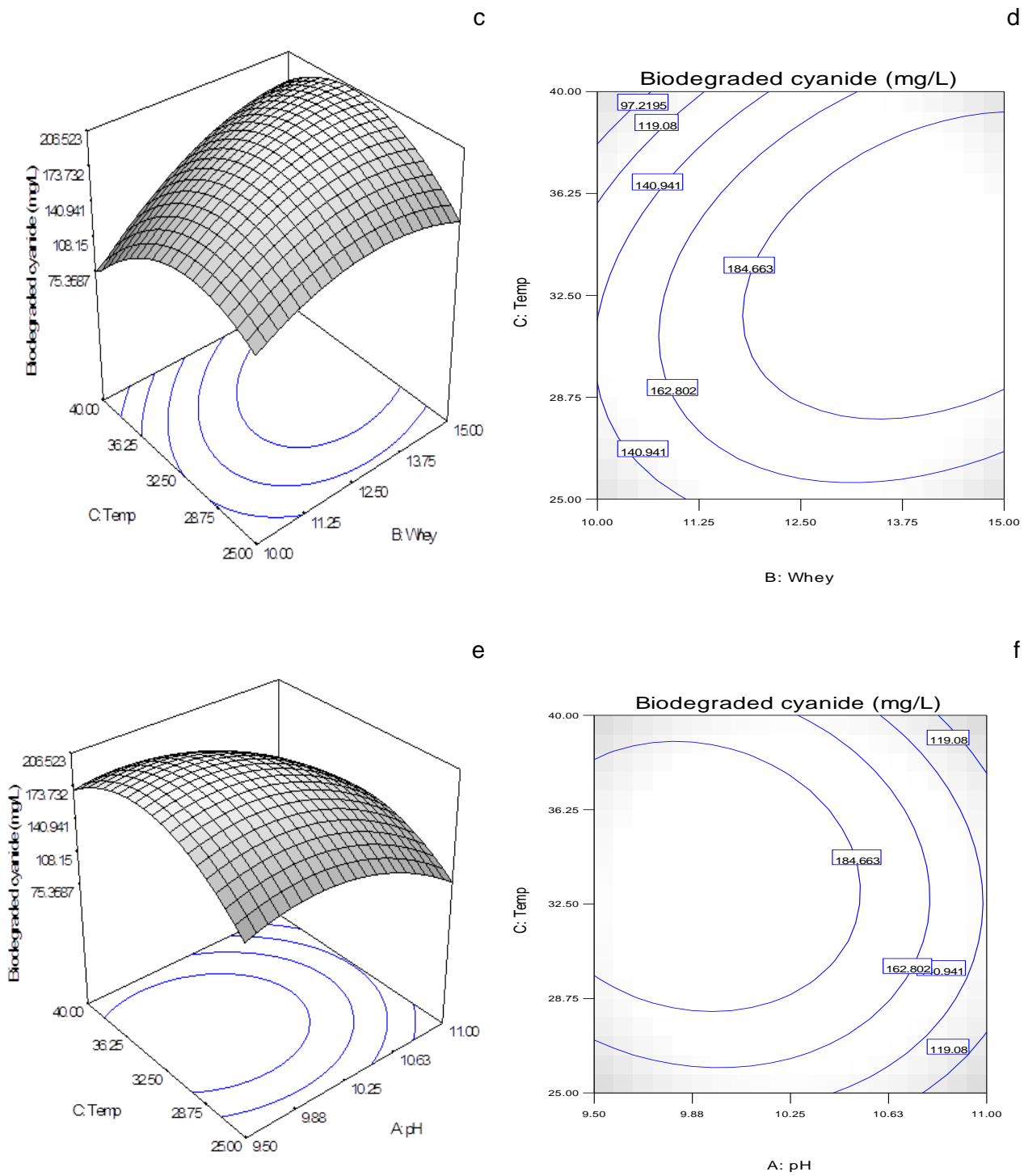


**Figure 4.18:** Normal probability plot of the studentised residuals

### 4.3.3.3 Graphical representation of the response surface model

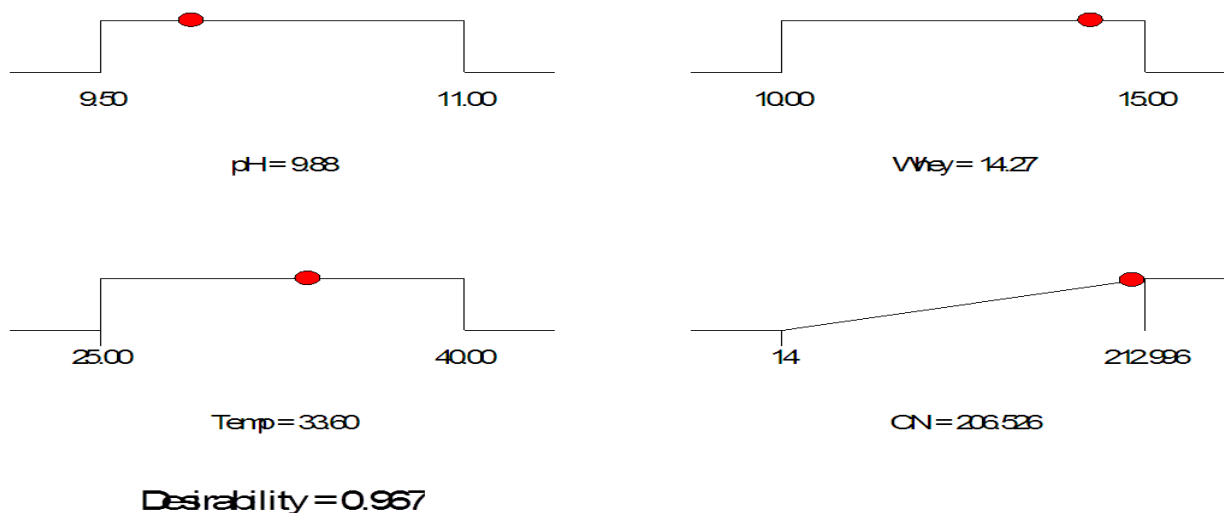
The interaction effects and optimal levels of the variables were determined by plotting the response surface curves. Figure 4.19 shows the 3D graphs of the interactions between cyanide biodegradation and the independent variables, that is, pH, temperature and whey concentration. Figure 4.19a and b show the 3D response surface and corresponding contour plots, respectively, of interactions between varying pH and whey concentration. The surface plot shows that with an increase in whey concentration, the cyanide biodegradation also increases. Figure 4.19c and d show the 3D response surface and corresponding contour plots, respectively, of interactions between varying temperature and whey concentrations, whereby an increase in both the variables increased the cyanide biodegradation. Figure 4.19e and f show the 3D response surface and corresponding contour plots, respectively, of interactions between varying temperatures and pH. The plots show that higher pH values will result in low degradation efficiency, while lower pH values might significantly increase the degradation, provided the temperature is above 30 °C but lower than 40 °C.





**Figure 4.19:** 3-D plots a, c, e and contour plots b, d, f plots showing the interactive effects of the independent variables on cyanide biodegradation

#### 4.3.3.4 Process optimisation



**Figure 4.20:** Numerical optimisation response for three independent variables: pH, whey-waste concentration and temperature

Design-Expert<sup>®</sup> Software allows for numeric optimisation of the assessed variables, where the input variables can either be set at maximum, minimum, target or within range. In this study, the response was set to maximise cyanide biodegradation and the resultant optimal responses for pH, temperature and whey concentration were found to be 9.88, 33.6 °C and 14.27 g/L, respectively –see Figure 4.20, with a maximum cyanide biodegradation of 206.526 mg F-CN/L (excluding cyanide volatilisation), from an initial concentration of 500 mg F-CN/L over an incubation period of 96 hours being achievable. A similar study observed that the isolated *Bacillus* species were more efficient at a temperature and pH of 31 °C and 10.3 respectively, an analysis obtained using a response surface methodology (Wu *et al.*, 2013), results similar to those obtained in this study.

#### 4.3.4 Summary

Independent variables such as pH, temperature and substrate (whey) concentration contribute significantly to cyanide degradability; hence the influence of these parameters was tested with the aim of optimising process efficiency. Using response surface methodology, the optimum conditions for pH, whey concentration and temperature were found to be 9.88, 14.27 g/L and 33.6 °C, respectively, with a maximum cyanide degradability of approximately 206 mg F-CN/L from an initial concentration of 500 mg F-CN/L over a period of 96 hours being achievable. A quadratic model was obtained using Design-Expert<sup>®</sup> 6.0. The model employed provided good quality predictions for the tested variables in terms of cyanide degradability and a good correlation coefficient of  $R^2$  0.914 was obtained. Using this model, we can predict the response of these variables at any point.

## **4.4 Continuous biodegradation of F-CN in a dual-stage packed-bed reactor**

### **4.4.1 Introduction**

Environmental legislation focusing on wastewater disposal in industries that utilise cyanide and/or cyanide-related compounds has become increasingly stringent worldwide. Many companies that utilise cyanide products are required to comply with the Cyanide International Code associated with the approval of process certifications and management of industries, thus enforcing the treatment or recycling of cyanide-contaminated wastewater (Akcil, 2002, Gibbons, 2005).

The widely utilised processes throughout the world for remediating cyanide-contaminated waters include: 1) alkaline chlorination, 2) hydrogen peroxide, and 3) sulphur dioxide oxidation (INCO process). However, the use of these methods over the years has declined, owing to high capital investment and the production of toxic end products that require further treatment, thus increasing the operational costs of the processes. The development and operation of a biological treatment process for cyanide containing wastewater at the Homestake Mine in 1984 provoked an interest from researchers in the biodegradation of cyanide. The process utilises microbial species such as bacteria and fungi (including algae) which possess different enzymes that are responsible for cyanide catalysis (Gupta *et al.*, 2010). The biological processes involved are environmentally friendly, as they do not produce toxic compounds (Dash *et al.*, 2009). As a result, a dual-stage packed-bed system that would facilitate cyanide biodegradation, which would be combined with a pneumatic bioreactor that would facilitate nitrification, was proposed.

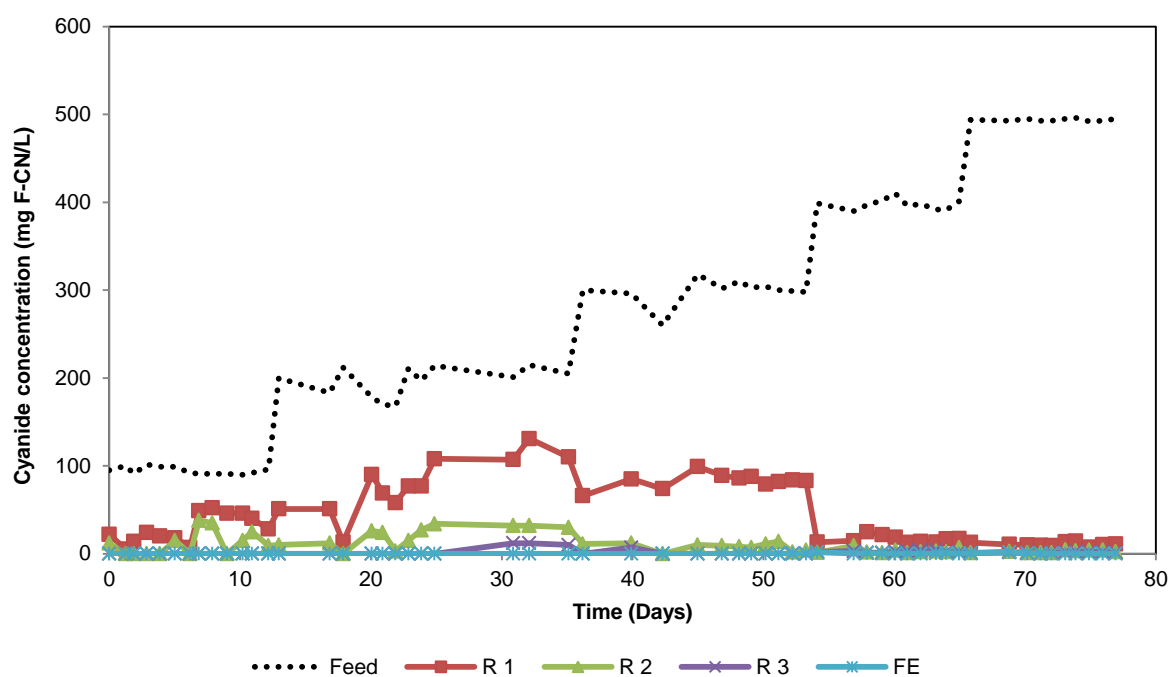
### **4.4.2 Aims and objectives**

The objectives were to:

- assess the ability of the CDB for continuous cyanide biodegradation at different cyanide concentrations in a constructed dual-stage packed-bed bioreactor, combined with a pneumatic bioreactor; and
- evaluate the nitrification and aerobic denitrification of the CDB in a continuous system.



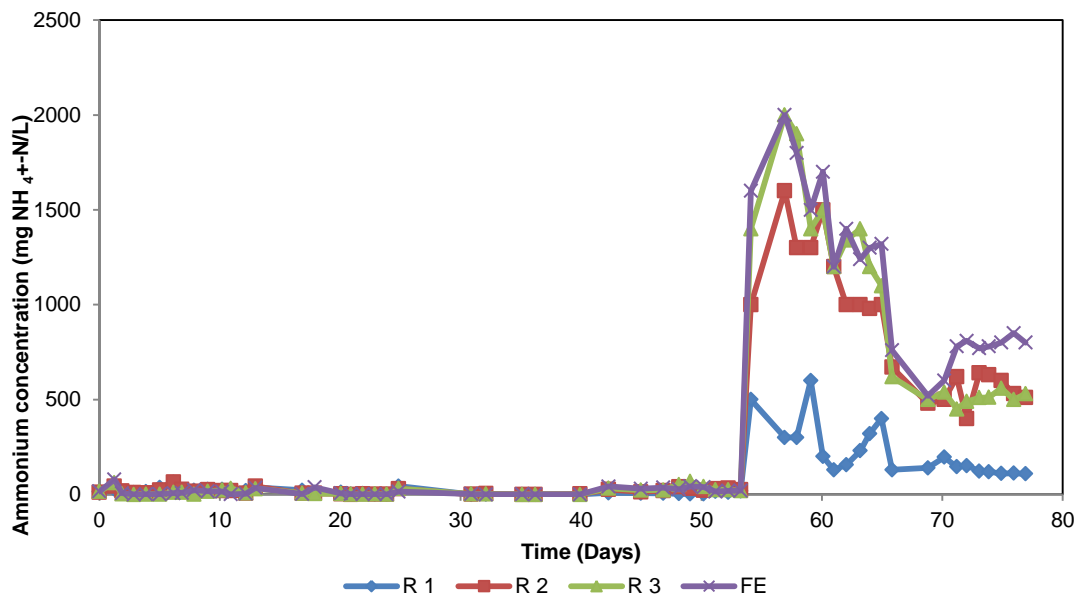
### 4.4.3 Results and discussion



**Figure 4.21:** Residual free cyanide concentration as a function of time and feed concentration

Dual-stage packed-bed reactors, operated at the optimised temperature (33.60 °C) and pH (9.88), were continuously fed with 100 mg F-CN/L during the start-up period. The residual cyanide concentration from Reactor 1 (R1), 2 (R2), 3 (R3) and the final effluent (FE) was analysed. The average biodegradation efficiency using 100 mg F-CN/L in R1, R2, R3 and FE was 72%, 86%, 100% and 100%, respectively. The feed CN<sup>-</sup> concentration was subsequently increased to 200 mg F-CN/L, where the average biodegradation efficiency from R1, R2, R3 and FE was found to be 61%, 90%, 99% and 100%, respectively. This state was maintained for a period of 10 days before increasing the F-CN concentration to 300 mg F-CN/L. The degradation efficiency increased in all the reactors, suggesting the biofilm was stable. The average degradation efficiency for R1, R2, R3 and FE was observed to be 72%, 97%, 99.8% and 100%, respectively. This was maintained for 14 days. During this period, significant biofilm growth on the reactor walls and in the granite rocks in the packed-bed reactors was observed. The biomass retention within the reactors led to higher effective cell concentration and thus higher degradation efficiencies. When the concentration was increased to 400 mg F-CN/L, the biofilm within the packed-bed reactors had already stabilised. This led to even higher degradation efficiencies as the degradation efficiency in R1, R2, R3 and FE was found to be 95%, 99%, 99% and 99.9%, respectively. The acclimatisation of the biofilm led to effective cyanide biodegradation efficiency. Similarly,

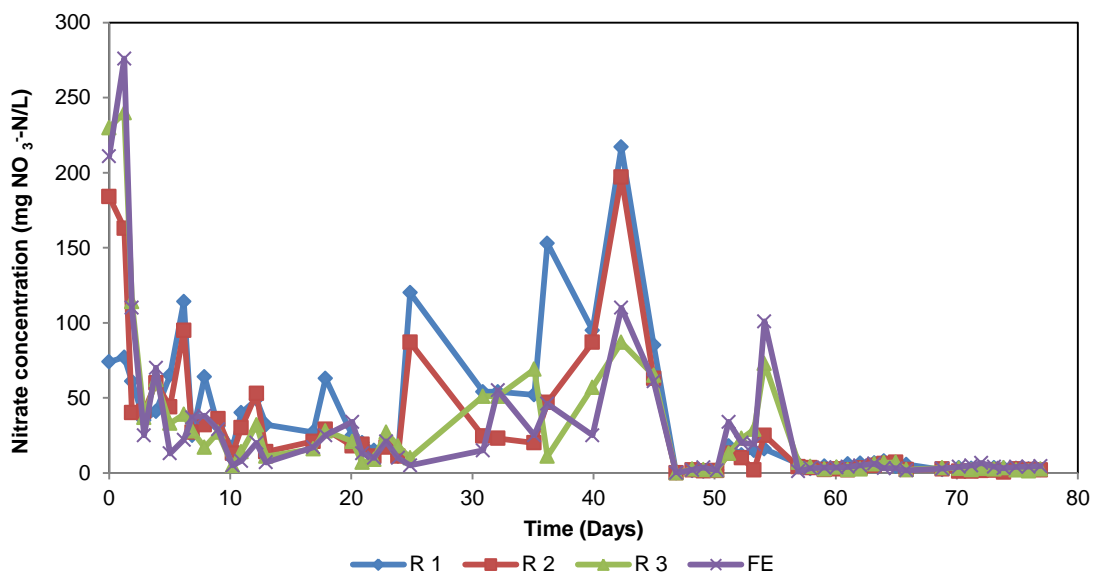
when the concentration was increased to 500 mg F-CN/L, the results were similar to that observed when the concentration was set at 400 mg F-CN/L. R1, R2, R3 and FE operated at 98%, 99.5%, 99.7% and 99.95% efficiency, respectively. The increased biodegradation rates coincided with the development of the attached microbial community, suggesting that this was a function of biomass retention rather than the activation of a particular metabolic pathway. From the data, it was observed that the microbial community was not inhibited at feed cyanide concentration of 500 mg F-CN/L, contradicting the maximum threshold of 200 mg F-CN/L for an active biological process that was set out by Kuyucak and Akcil (2013).



**Figure 4.22:** Ammonium concentration profile produced from cyanide biodegradation as a function time

Reactor 3 (R3), a pneumatic bioreactor, served as a nitrification reactor where the conversion of ammonium formed from cyanide biodegradation took place. The volume of R3 was 0.28 L with a working volume of 0.25 L, which was equivalent to a hydraulic retention time of approximately 24 hours. When cyanide concentration was set at 100 mg F-CN/L, the average residual ammonium concentration from R1, R2, R3 and FE was 25.2 mg NH<sub>4</sub><sup>+</sup>/L, 22.6 mg NH<sub>4</sub><sup>+</sup>-N/L, 14.4 NH<sub>4</sub><sup>+</sup>-N/L and 13.7 NH<sub>4</sub><sup>+</sup>-N/L, respectively. When the cyanide concentration was increased to 200 mg F-CN/L, the average residual ammonium concentration from R1, R2, R3 and FE was 11.1 NH<sub>4</sub><sup>+</sup>-N/L, 8.1 NH<sub>4</sub><sup>+</sup>-N/L, 7.1 NH<sub>4</sub><sup>+</sup>-N/L and 8.5 NH<sub>4</sub><sup>+</sup>-N/L, respectively. Similarly, it was expected that as the cyanide concentration increased, the rate of ammonium formation would also increase. Therefore, when the concentration of cyanide was increased to 300 mg F-CN/L, the average residual ammonium concentration from R1, R2, R3 and FE was found to be 7, 21.8, 28 and 24.7 NH<sub>4</sub><sup>+</sup>-N/L,

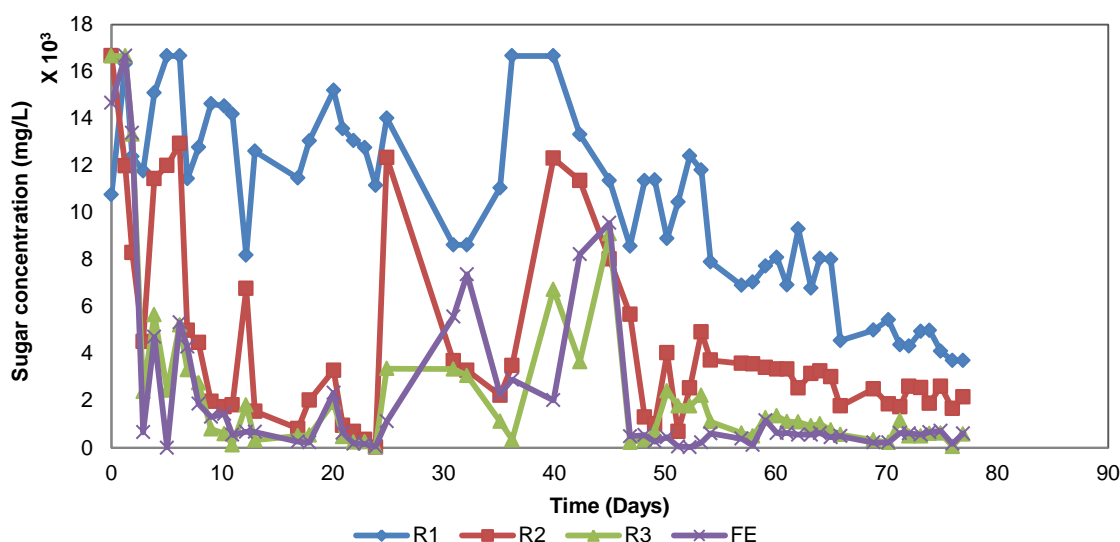
respectively. However, when the cyanide concentration was increased to 400 mg F-CN/L, high ammonium concentrations in all the reactors were observed as a result of high cyanide biodegradation rates, thus leading to accumulation of ammonium in the medium. The average residual ammonium concentration from R1, R2, R3 and FE was found to be 313.6 mg  $\text{NH}_4^+\text{-N/L}$ , 1188 mg  $\text{NH}_4^+\text{-N/L}$ , 1444 mg  $\text{NH}_4^+\text{-N/L}$  and 1506 mg  $\text{NH}_4^+\text{-N/L}$ , respectively. Similarly, the residual concentration was high when the cyanide concentration was set to 500 mg F-CN/L. The average residual ammonium concentration from R1, R2, R3 and FE was 133.8 mg  $\text{NH}_4^+\text{-N/L}$ , 558 mg  $\text{NH}_4^+\text{-N/L}$ , 521  $\text{NH}_4^+\text{-N/L}$  mg and 747 mg  $\text{NH}_4^+\text{-N/L}$ , respectively. These results demonstrate that as the cyanide concentration increased, the rate of ammonium formation also increased, as the biofilm within the system had stabilised, thus accelerating cyanide biodegradation to form ammonium. When the cyanide concentration was set at 100 to 300 mg F-CN/L, the microbial species were able to nitrify the formed ammonium, but when the concentration was increased from 400 to 500 mg F-CN/L, nitrification failed. Based on the reported nitrification ability of the *Bacillus* species employed in this study, an increase in the HRT in R3 to 48–72 hours could have resulted in complete removal of ammonium. This demonstrates the importance of setting the operational parameters in a bioprocess to achieve maximum degradation efficiencies.



**Figure 4.23:** Nitrate concentration profile produced from the nitrification stage as a function of time

During the startup of the experiment, the nitrate concentration in all the reactors, including the final effluent, was high. R1, R2, R3 and FE had an initial concentration of 74 mg  $\text{NO}_3^-\text{-N/L}$ , 184 mg  $\text{NO}_3^-\text{-N/L}$ , 230 mg  $\text{NO}_3^-\text{-N/L}$  and 211 mg  $\text{NO}_3^-\text{-N/L}$ , respectively (Fig. 4.23). From day 0 to day 24, the nitrate concentration decreased in all the reactors, indicating aerobic

denitrification by the cyanide-degrading bacteria. These results demonstrate that the microbial species were able to simultaneously utilise the ammonium and the formed nitrates. This phenomenon was observed by Chen *et al.* (2012) where the *Rhodococcus sp* CPZ24 was able to utilise the ammonium and nitrates simultaneously. Furthermore, the *Bacillus* species have been reported to be able to carry out nitrification and aerobic denitrification heterotrophically (Kim *et al.*, 2005, Yang *et al.*, 2011, Zhang *et al.*, 2012). From day 25 to day 50, the nitrate concentration in all the reactors increased, with the maximum nitrate concentration in R1, R2, R3 and FE on day 42 being 217 mg NO<sub>3</sub><sup>-</sup>-N/L, 197 mg NO<sub>3</sub><sup>-</sup>-N/L, 87 mg NO<sub>3</sub><sup>-</sup>-N/L and 110 mg NO<sub>3</sub><sup>-</sup>-N/L, respectively. From day 57 to day 78, the nitrates were utilised in the reactors, resulting in the final effluent concentrations that ranged from 1 to 6.5 mg NO<sub>3</sub><sup>-</sup>-N/L, thus meeting the World Health Organization nitrate threshold of 11 mg NO<sub>3</sub><sup>-</sup>-N/L (W.H.O., 2011). The low nitrate concentration observed from day 57 to day 78 might be due to the failure of the nitrification stage during this period, and partly as a result of aerobic denitrification by the microbial consortia.



**Figure 4.24:** Sugar metabolism during cyanide biodegradation

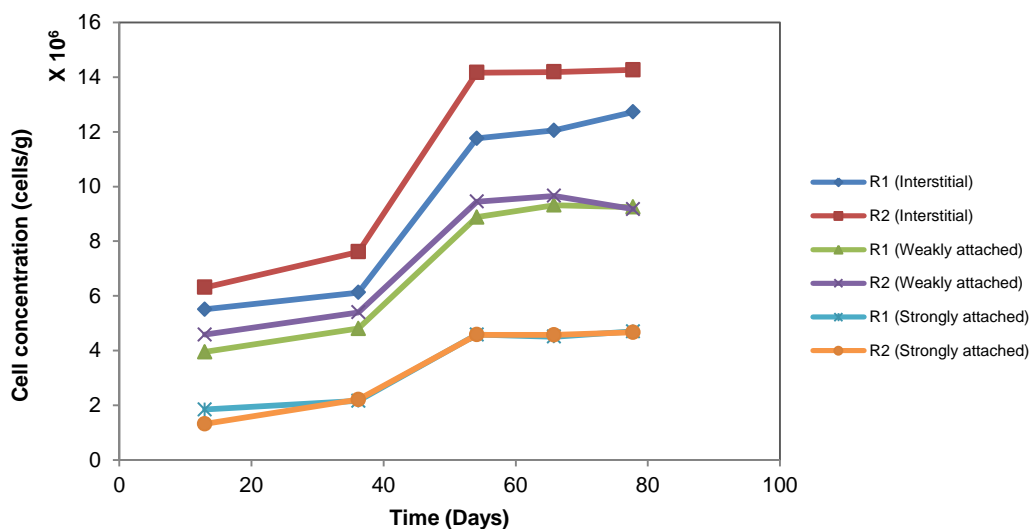
Sugar concentration during the experimental run was quantified using the method developed by Miller (1959), and was observed to fluctuate until day 52 as a result of metabolism by the microbial consortium (Fig. 4.24). From day 52 until the end of the experimental run, when the cyanide concentration was set at 400 to 500 mg F-CN/L, the sugar concentration decreased significantly in all the reactors. The high sugar metabolism experienced during this period might be due to the toxicity of cyanide, and as a form of resistance, the microbial species increased their sugar metabolism in order to survive. During this stress period, the microbial

species might have produced high concentrations of cyanide-degrading enzymes in response to the high cyanide concentration that the microbes experienced.

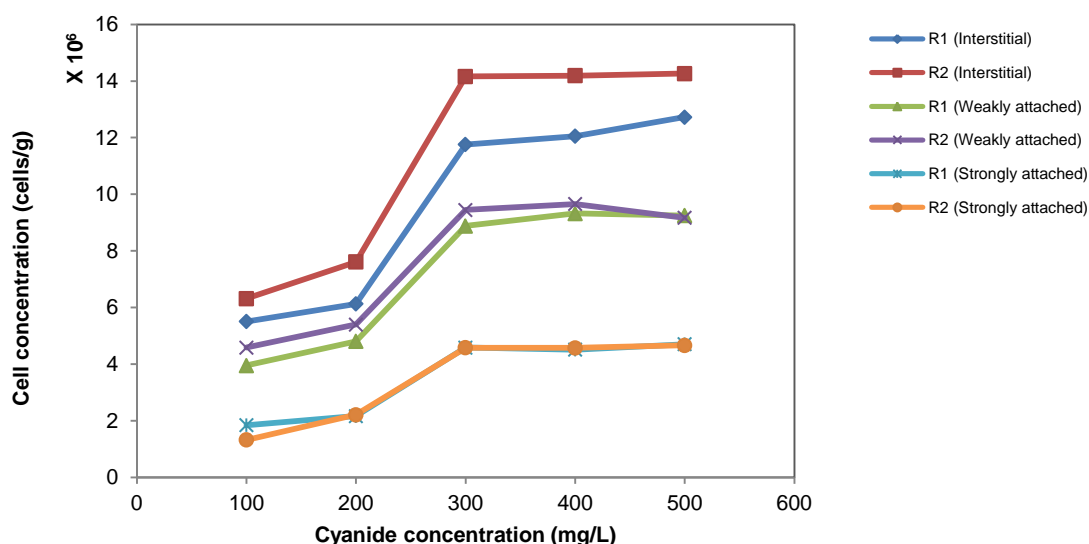
### Biofilm attachment on the PBB packing

It was proposed that during biofilm formation, the microbial population attaches to surfaces to form two distinctive phases: reversible, and firm or irreversible attachment (Van Loosdrecht *et al.*, 1990). The microbial cell numbers associated with the three detachment stages, that is, interstitial, weakly attached and strongly attached, were quantified intermittently before the cyanide concentration was increased (Fig. 4.25). The microbial growth within the reactors followed a logistic growth pattern with three distinctive growth phases being observed, viz., lag, exponential and stationary phases. The microbial species showed a similar growth pattern in all the bioreactors. From days 0 to 36, when the cyanide concentration was set between 100 to 200 mg F-CN/L, the microbial species showed a lag-phase period (R1 and R2), and thereafter the microbial population increased exponentially prior to a cyanide concentration increase to 300 mg CN/L. The microbial species reached the stationary phase between days 54 and 78. The maximum cell concentration observed as interstitial, weakly attached and strongly attached for R1 and R2 (primary units for cyanide biodegradation), were  $1.28 \times 10^7$  cells/g,  $9.30 \times 10^6$  cells/g,  $4.70 \times 10^6$  cells/g and  $1.42 \times 10^7$  cells/g,  $9.45 \times 10^6$  cells/g and  $4.67 \times 10^6$  cells/g, respectively.

a



b



**Figure 4.25:** Microbial concentration in the three detachment stages, viz., interstitial, weakly attached and strongly attached cells, as a function of (a) cyanide concentration and (b) time

From this information, it was observed that the microbial population attached to the packing was not inhibited by an increase in the cyanide concentration; overall, the microbial population was able to tolerate, thus degrade, the cyanide even at alleviated cyanide concentration of 500 mg F-CN/L (Fig. 4.21), although with reduced nitrification and denitrification capabilities, thus showing the suitability of the microbial consortia employed in this study.

#### 4.4.4 Summary

The alkaliphillic bacterial consortia dominated by *Bacillus* species, which were employed in this study, were determined to be cyanide degraders, nitrifiers and aerobic denitrifiers. Using the optimised data from batch cultures, a continuous process for the biodegradation of cyanide using a packed-bed system was designed. It was observed that the microbial species degraded cyanide successfully, with an overall degradation efficiency of >99.9 % irrespective of the cyanide-loading concentration. This was also confirmed by the stability of the detached microbial species at various cyanide concentrations (100 to 500 mg F-CN/L), as the microbial population did not show any decline in cell numbers. Furthermore, the organisms were able to carry out nitrification and aerobic denitrification, although nitrification was minimal when the cyanide concentration was increased from 400 to 500 mg F-CN/L. However, nitrification and denitrification rates can be improved by increasing the residence time of the nitrification and aerobic denitrification stage to optimise the efficiency of the bioprocess.

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**CHAPTER 5**  
**CONCLUSIONS**  
**AND**  
**RECOMMENDATIONS**

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## CHAPTER 5

### CONCLUSIONS AND RECOMMENDATIONS

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#### 5.1 Conclusion

The presence of cyanide in surface and groundwater reserves resulting from anthropogenic activities poses a huge threat to the environment and living organisms that feed from these water reserves. Wastewaters from the cyanidation process and electroplating facilities are normally within the alkaline range and are major contributors of cyanide in the environment. There have been numerous studies on the biological degradation of cyanide, but these studies have focused only on batch systems that contain low cyanide concentrations. Additionally, the microbial species were supported with refined nutrient sources and the nitrification of the formed ammonia had not been thoroughly explored. Therefore, the aim of this study was to isolate bacterial species that are resistant and capable of degrading cyanide, coupled with the bioremediation of the products formed from the biodegradation process.

Bacterial species were isolated from electroplating wastewater that contained significant cyanide concentrations from an electroplating plant located in Cape Town, Western Cape Province, South Africa. Mesophilic alkali-tolerant bacterial species were isolated and these isolates were found to belong to the *Bacillus* genus using the 16S rDNA gene; these species were then tested for cyanide degradation in nutrient broth. It was observed that these species are able to degrade high cyanide concentrations up to 500 mg F-CN/L and these species are referred to as cyanide-degrading bacteria (CDB). Additionally, the application of agro-waste products was assessed as potential supplements for the biodegradation process using waste materials of *Ananas comosus* (pineapple), *Ipomea batatas* (sweet potato) and *Beta vulgaris* (beetroot), spent brewer's yeast (SBY) and whey. Minimal cyanide degradation efficiencies were observed in all the tested waste materials except for the medium that was supplemented with whey, where high cyanide degradation took place. This exceeded the degradation efficiency that was observed in nutrient broth; hence whey was chosen as a suitable supplement. However, owing to high degradation efficiencies from the whey-supported media, elevated concentrations of ammonium were observed and as a result, nitrification and aerobic denitrification of ammonium and nitrates by the isolated bacterial species were evaluated.

Batch studies using the CDB and ammonia-oxidising bacteria (AOB) demonstrated that the CDB were more efficient in nitrification than the traditionally utilised AOB; these species were also observed to carry out aerobic denitrification while the AOB could not denitrify the formed nitrates. Highly aerated bioreactor systems such as the pneumatic bioreactor were observed to increase the nitrification performance. The CDB were also observed to be capable of



nitrification and aerobic denitrification in the presence of cyanide, where the presence of cyanide did not affect the nitrification process; however nitrification with AOB was significantly inhibited by cyanide concentrations above 1 mg F-CN/L. The robustness of the CDB in nitrification and aerobic denitrification was confirmed in cyanide containing wastewater in a pneumatic bioreactor through a fed-batch system consisting of 3 cycles, where over 99% treatment efficiency was observed.

The effect of independent variables such as temperature, pH and substrate concentration was evaluated using response surface methodology. Within RSM, central composite design was chosen and the optimum conditions for temperature, pH and substrate concentration were found to be 33.6 °C, 9.88 and 14.27 g/L, respectively. Using the optimised conditions, a continuous biodegradation process in a dual-stage packed-bed reactor coupled with a pneumatic bioreactor was employed. The concentration of cyanide was gradually increased from 100 to 500 mg F-CN/L in a period of approximately 80 days and it was observed that the alkali-tolerant organisms had an overall degradation efficiency of approximately  $\geq 99.9\%$ , irrespective of cyanide loading. This proved, beyond reasonable doubt, that these organisms are highly efficient in degrading cyanide-containing wastewater, even at high concentrations. However, although the organisms were able to nitrify the formed ammonium, when the concentration was set from 400 to 500 mg F-CN/L, nitrification failed as a result of high ammonium concentrations that were formed. The concentration of nitrates decreased over time, indicating aerobic denitrification by the bacterial consortium. Therefore, these mesophilic alkali-tolerant *Bacillus* species can be labelled as cyanide degraders, nitrifiers and aerobic denitrifiers.

The process that was employed in this study is environmentally friendly and sustainable, and typifies a process that can be utilised at a larger scale for overall cyanide degradation and the formed products i.e. ammonium and nitrates so that the water can be re-used.

## 5.2 Recommendations

The following recommendations are suggested for future research:

- A study on the mechanism of aerobic denitrification should be conducted.
- Consider an additional nitrification-aerobic denitrification bioreactor for effective nitrification and denitrification and optimise the residence time so that the water meets discharge levels.
- Determine how to control bio-fouling in the system as the system fouls over a period of time as a result of biofilm formation.

- The use of the gyrase gene is encouraged for the identification of *Bacillus* species as it differentiates these organisms at a species level.
- Increase the cyanide-loading concentration until the system destabilises so that systems' threshold concentration can be known.
- More attention should be directed to biodegradation of metal complexed cyanides, specifically, strong acid dissociable cyanides and subsequent biosorption of the released metals in continuous systems.

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## CHAPTER 6 REFERENCES

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## APPENDICES

### Appendix A: Analytical procedures

#### A1. Colometric assay for determination of reducing sugars

##### A1.1 Reagents

##### 3,5- Dinitrosalicylic acid (DNS) reagent

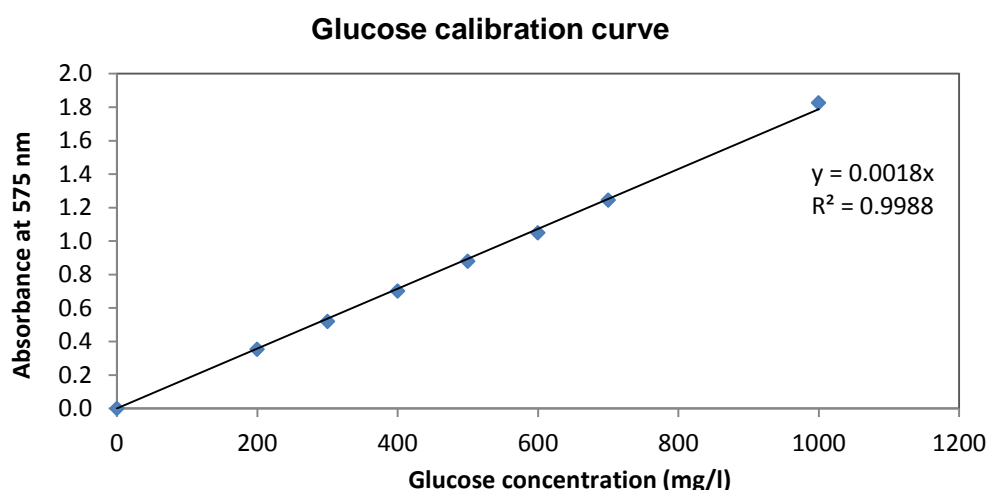
Dissolve 10 g dinitrosalicylic acid, 2 g phenol, 0.5 g sodium sulphite and 10 g sodium hydroxide in 1L distilled water.

##### 40% Potassium sodium tartrate

Dissolve 40 g of potassium sodium tartrate in 1L of distilled water.

##### A1.2 The glucose standard curve

A stock solution containing 1000 mg/L of glucose was prepared and a series of standard solutions was prepared by diluting the stock solution to 200, 300, 400, 500, 600, 700 and 1000 mg/L. The procedure was conducted according to Miller (1959).



**Figure A1.1:** Glucose calibration curve using the DNS method

##### A1.3 Calculation of reducing sugar concentration

The reducing sugar concentration in the samples was calculated from the standard curve, for example, if a sample that was diluted 100 times had an absorbance of 0.223:

$$\begin{aligned}\text{The sugar concentration} &= \frac{0.223}{0.0018} \times 100 \\ &= 12338.89 \text{ mg/L}\end{aligned}$$

#### A2. Cell count

##### A2.1 Cell count calculations

Depth of THOMA chamber = 0.02 mm

Width of one small square = 0.05 mm

Volume of one small square = depth x area

$$= 0.02 \text{ mm} \times (0.05 \text{ mm} \times 0.05 \text{ mm})$$

$$= 5 \times 10^{-5} \text{ mm}^3$$

$$= 5 \times 10^{-8} \text{ mL}$$

Number of small squares counted = 64

Volume of small squares counted =  $64 \times (5 \times 10^{-8} \text{ mL})$

$$= 0.0000032 \text{ mL}$$

Number of cells counted in the 64 squares = n

$$\text{Cell concentration (cells/mL)} = \text{dilution factor} \times \frac{n}{0.0000032 \text{ mL}} \times 1 \text{ mL}$$

$$= \text{dilution factor} \times n \times 312500$$

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