CONTINUOUS BIOREMEDIATION OF ELECTROPLATING EFFLUENT

by

BRUNO ALEXANDRE QUISTORP SANTOS

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Supervisor: Dr Seteno Karabo Obed Ntwampe
Co-supervisor: Dr Gift Muchatibaya

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Signature

30 August 2013

Date
There are significant quantities of free cyanide (F-CN) and heavy metal contaminated effluent being discharged from electroplating operations globally. However, there is an overwhelming tendency in the industry to use physical and/or chemical treatment methods for cyanides (CNs) and heavy metals in effluent. Although these methods may be effective for certain CNs and heavy metals, they produce toxic by-products and also involve high operational and capital investment costs when compared to bioremediation methods. In this study, the design of a two-stage membrane bioreactor (MBR) system was conceptualised for the bioremediation of CNs and heavy metals in the effluent which was collected from an electroplating facility located in the Western Cape, South Africa. The design included a primary inactive bioremediation stage, to reduce the impact of contaminant concentration fluctuations, and a secondary active bioremediation stage, to remove the residual contaminants, in the effluent under alkaline pH conditions which typify most industrial effluent containing these contaminants. An analysis of the electroplating effluent revealed that the effluent contained an average of 149.11 (± 9.31) mg/L, 5.25 (± 0.64) mg/L, 8.12 (± 4.78) mg/L, 9.05 (± 5.26) mg/L and 45.19 (± 25.89) mg/L of total cyanide (T-CN), F-CN, weak acid dissociable cyanides (WAD-CNs), nickel (Ni), zinc (Zn) and copper (Cu), respectively.

An Aspergillus sp., which displayed the characteristic black conidiophores of the Aspergillus section Nigri, was isolated from the electroplating facilities’ effluent discharge using a selective pectin agar (PA) and subcultured on 2% (v/v) antibiotic (10,000 units/L penicillin and 10 mg streptomycin/mL) potato dextrose agar (PDA). The isolate was tolerant to F-CN up to 430 mg F-CN/L on F-CN PDA plates which were incubated at 37 °C for 5 days. However, a significant decline in microbial growth was observed after 200 mg F-CN/L, thus indicating that the isolate was suitable for the bioremediation of the electroplating effluent. The identification of the isolate as Aspergillus awamori (A. awamori) was definitively determined using a multi-gene phylogenetic analysis, utilising ITS (internal transcribed spacer), β-tubulin and calmodulin gene regions. Although an anomaly in the morphology of the conidia of the isolate was observed during the morphological analysis, indicating a possible morphological mutation in the isolate. A comparative study between “sweet orange” (Citrus sinensis (C. sinensis)) pomace, “apple” (Malus domestica (M. domestica)) pomace, “sweetcorn” (Zea mays (Z. mays)) cob and “potato” (Solanum tuberosum (S. tuberosum)) peel, i.e. waste materials considered to be agricultural residues, was conducted in order to assess their potential and as a sole carbon source supplement for A. awamori biomass development for the bioremediation of CNs and heavy metals.
The suitability of these agricultural residues for these activities were as follows: C. sinensis pomace > M. domestica pomace > Z. mays cob > S. tuberosum peel. For purpose of the sensitivity analysis, a temperature range of 20 to 50 °C and an alkaline pH range of 7 to 12 showed that: (1) optimal conditions for the uptake of Ni, Zn and Cu occurred at pH 12 and a temperature of 37.91 and 39.78 °C using active and inactive A. awamori biomass and unhydrolysed and hydrolysed C. sinensis pomace, respectively; (2) F-CN conversion increased linearly with an increase in pH and temperature using unhydrolysed and hydrolysed C. sinensis pomace; and (3) optimal conditions for the F-CN conversion and the respective by-products and sugar metabolism using active A. awamori biomass occurred at 37.02 °C and pH 8.75 and at conditions inversely proportional to F-CN conversion, respectively. The heavy metal affinity was Ni > Zn > Cu for all the biomaterials used and with the heavy metal uptake capacity being inactive A. awamori biomass > active A. awamori biomass > hydrolysed C. sinensis pomace > unhydrolysed C. sinensis pomace, respectively. Hydrolysed C. sinensis pomace had a 3.86 fold higher conversion of F-CN compared to the unhydrolysed C. sinensis pomace. The use of C. sinensis pomace extract as a nutrient media, derived from the acid hydrolysis of C. sinensis pomace, showed potential as a rich carbon-based supplement and also that low concentrations, < 0.1% (v/v), were required for the bioremediation of CNs and heavy metals.

The two-stage MBR system was operated at 40 °C since this temperature was conducive to the bioremediation of CN and heavy metals. The primary bioremediation stage contained hydrolysed C. sinensis pomace while the secondary bioremediation stage contained active A. awamori biomass, supplemented by the C. sinensis pomace extract. After the primary and secondary bioremediation stages, 76.37%, 95.37%, 93.26% and 94.76% (primary bioremediation stage) and 99.55%, 99.91%, 99.92% and 99.92% (secondary bioremediation stage) average bioremediation efficiencies for T-CN, Ni, Zn and Cu were achieved. Furthermore, the secondary bioremediation stage metabolised the CN conversion by-products with an efficiency of 99.81% and 99.75% for formate (CHOO−) and ammonium (NH4+), respectively. After the first, second and third acid regeneration cycles of the hydrolysed C. sinensis pomace, 99.13%, 99.12% and 99.04% (first regeneration cycle), 98.94%, 98.92% and 98.41% (second regeneration cycle) and 98.46%, 98.44% and 97.91% (third regeneration cycle) recovery efficiencies for Ni, Zn and Cu were achieved. However, the design only managed to treat the effluent for safe discharge and the use of a post-treatment stage, such as reverse osmosis, is recommended to remove the remainder of the trace contaminants and colour from the effluent to ensure that the effluent met the potable water standards for reuse. There was a relatively insignificant standard deviation (≤ 3.22%) detected in all the parameters measured in the continuous operation and this indicates the reproducibility of the bioremediation efficiency in this continuous system.
RESEARCH OUTPUTS

The following research outputs represent contributions by the candidate to scientific knowledge and development during his master’s candidacy (2012–2013):

- **Publications**


- **Presentations**


Posters

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### LIST OF SYMBOLS

**Nomenclature**

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<td>Xi</td>
<td>Coded independent variables</td>
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<tr>
<td>Yi</td>
<td>Response variable</td>
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**Greek symbols**

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**Subscripts**

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ABBREVIATIONS

A. awamori  Aspergillus awamori
A. niger  Aspergillus niger
C. sinensis  Citrus sinensis
CCD  Central composite design
CHOO  Formate
CN  Cyanide
Cu  Copper
CYA  Czapek yeast agar
F-CN  Free cyanide
Fe  Iron
FTIR  Fourier Transform Inferred
ICP-AES  Inductively coupled plasma atomic emission spectroscopy
ITS  Internal transcribed spacer
M. domestica  Malus domestica
MBR  Membrane bioreactor
MEA  Malt extract agar
Ni  Nickel
NIST  National Institute of Standards and Technology
NH₄⁺  Ammonium
PA  Pectin agar
PCR  Polymerase chain reaction
PDA  Potato dextrose agar
RSM  Response surface methodology
S. tuberosum  Solanum tuberosum
SAD-CN  Strong acid dissociable cyanide
T-CN  Total cyanide
TDS  Total dissolved solids
TRS  Total reduced sugars
UF  Ultrafiltration
WAD-CN  Weak acid dissociable cyanide
Z. mays  Zea mays
Zn  Zinc
CHAPTER 1
INTRODUCTION
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INTRODUCTION

1.1. Background

Electroplating is a process whereby a thin layer of a metal is coated onto an object (workpiece) by the electrolytic decomposition of a metal salt, such as WAD-CNs and/or strong acid dissociable cyanides (SAD-CNs), in a solution. CN-based operations produce effluent containing significant concentrations of CN and heavy metal contaminants (Cushnie & CAI Resources Inc., 2009). As a result of the bio-accumulative nature of these toxic contaminants, research is currently being conducted to assess the feasibility of various processes with which to treat the contaminated effluent. However, the most popular method involves using conventional chemical and/or physical methods for the treatment of CN and heavy metal bearing effluent as compared to bioremediation methods. Nevertheless, in view of the high capital investment and operational costs involved, many industries either do not treat their effluent or else they treat it only partially. This is as a result of the fact that the municipal discharge standards of industrial effluent are not being properly monitored in South Africa.

Similarly, solid waste generation in South Africa is problematic with the majority of landfill sites reaching their maximum capacity. Approximately 427 million tonnes of solid waste is generated in South Africa annually, of which 40% (w/w) is organic waste (Greben & Oelofse, 2009). The average amount of solid waste generated per person is 0.700 kg/annum in South Africa. This is close to that of developed countries, for example 0.723 kg/annum in the United Kingdom and 0.870 kg/annum in Singapore, but more than that for developing countries, for example 0.300 kg/annum in Nepal (Greben & Oelofse, 2009). The application of biomaterials, such as agricultural residue and microbial biomass, is an emergent process for the removal of heavy metals in effluent, where a variety of heavy metal contaminants have been successfully removed from effluent utilising biomaterials (Wang & Chen, 2009; Sud et al., 2008). In addition, the use of some microorganisms which possess enzymatic mechanisms to convert CNs in effluent has also been studied (Gupta et al., 2010; Rao et al., 2010). The filamentous aerobic fungi of the Aspergillus section Nigri are commonly known for their ability to break down and utilise complex sugars, such as the lignocellulose present in agricultural residues, to form simpler components of the sugars to support its metabolic activities (Papagianni, 2007). In addition, they also possess the ability to bioremediate CNs and heavy metals (Rao et al., 2010; Wang & Chen, 2009).
However, the use of agricultural residues, particularly those residues that contain free hydroxyl functional groups, as pseudo-catalysts for CN conversion has not been reported in literature. Although, the use of agricultural residues has shown huge potential as a feedstock for the cultivation of microorganisms and/or production of enzymes that may be used to catalyse various processes, there are limited studies showing the use of these agricultural residues for the conversion of CNs and the removal of heavy metals (Nigam & Pandey, 2009; Li et al., 2008; Papagianni, 2007; Mitchell et al., 2006).

Since the early 1970s, there has been progress in developing economically viable continuous MBR processes (Gondongwana, 2007). In certain instances, multiple stages are used to control transfer rates and, thus, to improve the bioremediation of the contaminants. The selection of a bioremediation system, including its components, may provide improved conversion rates of certain components, while being restrictive to others, and prevent certain components from rendering the catalyst/pseudo-catalyst inactive as a result of the accumulative effects of contaminants in the system and/or prevent the catalyst/pseudo-catalyst from leaving the bioreactor (Fogler, 2006). These are some of the design parameters which must be monitored when developing an environmentally friendly process for the combined bioremediation of CNs and heavy metals.

Accordingly, the integration of a system utilising agricultural residues and microorganisms for the bioremediation of the CNs and heavy metal from effluent under alkaline conditions, which typify most industrial effluent containing these contaminants, has been demonstrated in a few studies only and, thus, it is desirable that a study be undertaken to demonstrate this (Gupta et al., 2010; Rao et al., 2010; Wang & Chen, 2009; Mitchell et al., 2006).

1.2. Problem statement

At present, electroplating companies discharge their effluent through municipal systems with either minimal or no treatment of CNs and heavy metals. Furthermore, there is a lack of a suitable bioprocess which may be used on a large scale to treat CNs and heavy metals and which is solely supported using agricultural residue as a primary carbon source under alkaline pH conditions.

Therefore, it is proposed that a suitable bioremediation process be designed for the continuous bioremediation of electroplating effluent to an acceptable standard so that the effluent can either be reused or to ensure that it is in compliance with the regulatory municipal discharge standards.
1.3. Hypothesis

It is hypothesised that a two-stage MBR process, comprising a primary inactive bioremediation stage, using an agricultural residue, in combination with a secondary active bioremediation stage, using a suitable Aspergillus section Nigri isolate, may efficiently bioremediate electroplating effluent for either safe municipal discharge or reuse with minimal residual by-products in the treated effluent recovered from the process.

1.4. Research objectives

The following specific research objectives were identified:

1. To profile the concentrations of the CNs and heavy metals identified in the effluent collected from an electroplating facility.
2. To bio-prospect, isolate and identify a suitable Aspergillus section Nigri isolate which would exhibit CN tolerance and the required conversion capabilities necessary for the bioremediation of electroplating effluent.
3. To assess the suitability of various agricultural residues to supplement the isolate as the sole primary carbon source in the presence of CN and heavy metal concentrations similar to those found in the electroplating effluent collected and select one as a suitable agricultural residue.
4. To analyse the effect of temperature and alkaline pH on CN conversion, the metabolism of CN conversion by-products and heavy metal uptake by active and inactive biomass of the isolate cultivated using the selected agricultural residue extract and the unhydrolysed and hydrolysed agricultural residue.
5. To design and operate a unique and continuous two-stage MBR process for the bioremediation of collected electroplating effluent for safe municipal discharge or reuse.

1.5. Significance of the study

This study will develop a two-stage MBR process for the continuous bioremediation of electroplating effluent containing CNs and heavy metals. This integrated process should provide both a feasible and an alternative solution that may be implemented on a medium to large scale in South African electroplating facilities for the bioremediation of effluent generated. This study should also demonstrate the potential of agricultural residues as a suitable material and feedstock for active biological processes and also highlight the low reagent requirements associated with such a process.
1.6. Delineation of the study

Several factors that were not considered in this study include the following:

- Scale-up experiments and economic feasibility of the proposed design
- Mathematical modelling of the following parameters:
  - Biosorption/bioaccumulation kinetics of heavy metals.
  - CN conversion kinetics.
  - CN conversion by-products metabolism kinetics.
  - Growth kinetics of the isolate.
CHAPTER 2
LITERATURE REVIEW
CHAPTER 2
LITERATURE REVIEW

2.1. Introduction

This chapter focuses on the background to and challenges encountered in the conventional physical and chemical treatment of CNs and heavy metals. The chapter will discuss the chemistry behind the treatment of CNs and heavy metals in effluent from CN-based electroplating operations. In addition, the chapter will highlight the beneficial and unique qualities of biomaterials and MBRs with an emphasis on their application for treating these environmental pollutants.

2.2. Electroplating

2.2.1. Background

Electroplating products are used in a variety of industries such as the automotive, shipping, airfreight, machinery, electronics, jewellery and defence. Reasons for using electroplating include improving the aesthetic appearance and enhancing properties and/or resistance to corrosion/wear. However, the core components of an electroplating process are the surface treatment and the electrolytic cell (Lou & Huang, 2006). Both before and after plating it is essential that the surface of the workpiece be treated in order to remove contaminants, as these may interfere with the bonding of metals on the workpiece’s surface and may result in either the poor deposition of the metal or else prevent it entirely. In addition, it is also vital to remove the residues after the plating process as they may be toxic. The surface treatment of workpieces includes the following (Lou & Huang, 2006):

- **Chemical cleaning** – the use of acid and/or alkaline solutions to remove heavy scaling and/or dirt, solid soil and greases on the surface of the workpiece. These processes may be improved by passing a current through and/or increasing the temperature in the cleaning baths.
- **Mechanical cleaning** – the polishing to remove surface imperfections. This is then followed by buffering to smooth the surface using the finer abrasives on the workpiece.
- **Surface modification** – the use of either coating with a metallic layer and/or hardening of the workpiece to improve the plating process.
- **Rinsing** – the use of water to remove the residues after cleaning or plating the workpiece. The effluent is then discharged into the municipal discharge system.
In an electrolytic cell, the electrodes (an anode and cathode) are immersed in a bath filled with an electrolyte and connected to a power source (Lou & Huang, 2006). The workpiece acts as a negatively charged cathode while the positively charged anode completes the electric circuit. The anode may be of two types: (1) sacrificial anodes are made from the metal/alloy that is to be plated onto the workpiece; or (2) inert anodes are inert, usually carbon, and serve only to complete the electrical circuit. Inert anodes do not provide fresh metal/alloy to replace that which has been removed from the solution to plate the workpiece’s surface (Lou & Huang, 2006; Kanani, 2004).

The electrolyte serves as the medium (electrical conductor) for the movement of ionic species which usually consist of: (1) aqueous solution of acids, alkalis and/or metal salts; (2) use of some agents to increase the electrolytic conductivity, bath stabilisers, levelling and metal distribution and wetting agents; and/or (3) optimising the chemical, physical or technological processes of the coating to improve the corrosion resistance, brightness or reflectivity, hardness, mechanical strength, ductility, internal stress and/or wear resistance (Lou & Huang, 2006; Kanani, 2004; Lowenheim, 1997). The properties of the electrolyte may be summarised as follows (Lou & Huang, 2006):

- **Electrolytic conductance** – the effect that inorganic and/or organic salts, acids and alkalis have on the degree of dissociation, mobility of ions, temperature, viscosity and electrolyte composition.
- **Covering power** – the ability of the electrolyte to form a uniform layer over the surface of the workpiece which is affected by the workpiece’s surface characteristics, temperature, viscosity, electrolyte composition and current density.
- **Macrothrowing power** – the ability of the electrolyte to lay a uniform surface coverage and thickness over the workpiece which is affected by the current distribution and density, electrolytic conductance, covering power and electrolyte agitation.
- **Microthrowing power** – the extent in which the electrodeposition occurs in cracks and valleys in the workpiece’s surface and which may be improved by activating the base of the cracks or valleys, thus inhibiting the outer surfaces from electrodeposition.

When an electric current is applied to the electrolytic cell by a power source, the positive ions in the electrolyte will gravitate towards the cathode while the negatively charged ions will gravitate towards the anode. The migration of the electrons into the anode, through the wiring and power source, and then out of the cathode, constitute the current. The positively charged metallic ions in the metallic salt in the electrolyte are attracted to the negatively charged workpiece (cathode) and are deposited onto its surface (Lou & Huang, 2006).
2.2.2. Types of metal coating

2.2.2.1. Sacrificial coatings

Sacrificial coatings, usually Zn and chromium, are used for the protection of the base, commonly iron (Fe) and steel, since the anodic nature of the binding to the base ensure that they are able to sacrifice themselves in order to protect the workpiece from corrosion (Lou & Huang, 2006).

2.2.2.2. Alloy coatings

Alloy coatings are the most commonly used plating operation and involve plating a workpiece in a solution with more than one type of metal, usually gold–Cu–cadmium, Zn–Fe, Zn–Ni, brass (Cu–Zn), bronze (Cu–tin), and tin–cobalt, in order to improve aesthetic appeal and/or in the interests of corrosion and wear resistance (Lou & Huang, 2006).

2.2.2.3. Decorative protective coatings

Decorative protective coatings, usually Cu, Ni, chromium and tin, are used for enhancing ascetic appeal and applying protection onto the workpiece (Lou & Huang, 2006).

2.2.2.4. Engineering coatings

Engineering coatings, usually gold, silver, ruthenium, rhodium, palladium, osmium, iridium, platinum, tin and lead, are used for enhancing surface properties, wear resistance, reflectivity and/or conductivity (Lou & Huang, 2006).

2.2.2.5. Multilayered coatings

Multilayered coatings are the succession of plating individual metals on a workpiece which results in the deposition of individual layers of each metal, such as, firstly, Cu, secondly, Ni and, thirdly, chromium, in that order, in order to improve aesthetic appeal, corrosion and wear resistance and/or weight saving (Lou & Huang, 2006).

2.2.2.6. Unusual metal coatings

Unusual metal coatings are rarely used in electroplating operations. However, there are three categories of these unusual metal coatings, namely platable metals from: (1) aqueous solutions using metals such as arsenic, bismuth and rhenium; (2) organic electrolyte using metals such as aluminium; and (3) fused-salt electrolyte using metals such as titanium, vanadium, molybdenum and tungsten for very specific purposes (Lou & Huang, 2006).
2.2.2.7. Minor metal coatings

Minor metal coatings, usually Fe, cobalt and indium, are easily plated metals with limited applications in industrial operations (Lou & Huang, 2006).

2.2.2.8. Conversion coatings

Conversion coatings are the plating of metal on the workpiece by reaction with a solution, such as chromate coatings by the reaction with aqueous chromic acid and aluminium, Zn, cadmium or magnesium workpiece, to improve corrosion resistance (Lou & Huang, 2006).

2.3. Agricultural residues

2.3.1. Classification and annual generation

Agricultural residues may be obtained from (1) cereals, such as maize, oats and sorghum; (2) roots and tubers, such as potatoes, cassava and yams; (3) oilseeds and pulses, such as soybeans, shelled groundnuts, sunflower and mustard seeds; and (4) fruits and vegetables, such as oranges, apples, tomatoes and onions. These residues are generated in the five stages of the food production chain, namely (Gustavsson et al., 2011):

- **Agricultural production** – waste generated as a result of mechanical damage or spillage during the harvest.
- **Post handling and storage** – waste generation as a result of spillage and damage during handling, storage and transportation between the farm and a distribution station.
- **Processing** – waste generated as a result of spillage, sorting out of unsuitable produce and residues from the produce.
- **Distribution** – waste generated during transportation to various markets.
- **Consumption** – waste generated by the consumption of agricultural produce by households.

Figures 2.1 and 2.2 present the percentage contribution after each food supply chain stage and the annual quantities that each of the agricultural residues generate on various continents. On an average mass basis, 11.07%, 7.79%, 10.48%, 5.68% and 10.14% of the cultivated agricultural products generate residues from the agricultural production, post-handling and storage, processing, distribution and consumption food production stages, respectively. Cereals, roots and tubers, oilseeds and pulses and fruits and vegetables generate, on average, 2400, 825, 575 and 1769 million tonnes of waste/annum globally, respectively (Gustavsson et al., 2011).
Figure 2.1: Percentage contribution of (i) cereals, (ii) roots and tubers, (iii) oilseeds and pulses and (iv) fruits and vegetables residues generated after each food production chain stage

(Gustavsson et al., 2011)
2.3.2. Treatment methods for agricultural residue

2.3.2.1. Combustion

Combustion is the oldest, simplest and most commonly used treatment process for producing thermal energy for various operations using organic materials. This process utilises an oxygen-rich environment at elevated temperatures above 1500 °C to produce H₂, CO₂, CO, SOₓ, NOₓ, steam and char. The efficiency of the conversion of the feedstock to CO₂ is largely dependent on the amount of oxygen supplied with an insufficient supply of oxygen resulting in both incomplete combustion and an elevated production of CO (Potgieter, 2011; Werthera et al., 2000).

2.3.2.2. Pyrolysis

Pyrolysis is the thermal decomposition of organic materials at temperatures between 350 and 600 °C in the absence of oxygen to produce syngas (H₂, CO₂, CO, CH₄ and H₂), SOₓ, NOₓ, steam, low molecular weight hydrocarbons and char. There are three types of pyrolysis processes (Potgieter, 2011; Yanik et al., 2007):

- **Slow pyrolysis** – in a limited oxygen environment to produce a higher yield of char and lower yields of light hydrocarbons.
- **Fast pyrolysis** – in the absence of oxygen to produce higher yields of light hydrocarbons and a lower yield of char.
- **Vacuum pyrolysis** – in a vacuum to ensure limited quantities of oxygen. However, this type of pyrolysis is unpopular at industrial levels as a result of the high capital and operational costs required to maintain the vacuum.
2.3.2.3. Gasification

Gasification is the thermal decomposition of organic materials using controlled quantities of air, oxygen and/or steam at temperatures above 800 °C to produce syngas (H₂, CO₂, CO, CH₄ and H₂), SOₓ, NOₓ, steam, high molecular weight hydrocarbons and char. However, the production of high molecular weight hydrocarbons may cause operational problems in the process and, thus, it is essential that the production of such hydrocarbons be limited (Potgieter, 2011; Malatji, 2009).

2.3.2.4. Conventional fermentation

Conventional fermentation refers to the utilisation of organic materials to produce value-added products such as enzymes, for example cellulase and pectinase, and chemicals, for example organic acids and alcohols (Nigam & Pandey, 2009; Mitchell et al., 2006).

2.3.2.5. Anaerobic digestion

Anaerobic digestion is the microbial conversion of organic materials into biogas (CH₄, CO₂, H₂S, N₂ and H₂) and undigested organic material in the absence of oxygen, using a mixed culture of microorganisms in a syntrophic process. Anaerobic digestion comprises of the following four stages (Greben & Oelofse, 2009; Nigam & Pandey, 2009):

- **Hydrolysis** – the breaking down of complex polymers into monomers by enzymes produced by the microorganisms.
- **Acidogenesis** – the fermentation of the monomers into organic and fatty acids, alcohols, H₂ and CO₂.
- **Acetogenesis** – the fermentation of the organic and fatty acids to acetic acid, CO₂ and H₂.
- **Methanogenesis** – the fermentation of acetic acid and H₂ to CH₄ and CO₂.

2.4. Membrane bioreactor technology

2.4.1. Background

A membrane is generally defined as a selective barrier which has the ability to control mass transfer between two bulk phases. Materials used in the construction of membranes include organic, non-organic, homogeneous or heterogeneous solids. The movement of a phase across the membrane is due to the presence of one or more driving forces that may be a gradient in concentration, pressure, temperature or electrical potential (Perry et al., 1988).
Membrane processes are categorised according to their pore size, molecular weight cut-off size and the pressure at which they operate (De Jager, 2009; Perry et al., 1988). Membrane separation processes may be broadly categorised into five groups, based on the pore size of the membranes (see Figure 2.3).

![Figure 2.3: Filtration spectrum (Perry et al., 1988)](image)

The main issue encountered in membrane operations is the fouling, which is the build-up of solutes on the membrane surface. This, in turn, inhibits the transport of fluid across the membrane (see Figure 2.4).

![Figure 2.4: Fouling on membrane](image)
The large surface area of membranes to their small volume allows for high operational capacity, a positive property with regards to industrial applications, especially MBRs (De Jager, 2009; Charcosset, 2006). The selection of membrane utilised in a MBR may provide either a barrier to limit the transport of certain components, while being permeable to others so as to prevent certain components from contacting a biocatalyst(s) in the bioreactor and/or prevent biocatalyst(s) leaving the bioreactor (Fogler, 2006).

2.4.2. Membrane modes of operation

2.4.2.1. Closed mode

Closed mode refers to a situation in which the feed may enter the shell or lumen, permeate through the membrane and exit the lumen or shell in a continuous stream, with no retentate stream. The trans-membrane pressure on the feed side is greater than on the permeate side, thus, initiating the transport of the fluid across the membrane (Bruining, 1989; Perry et al., 1988; Thakaran & Chau, 1986). However, this mode of operation is prone to excessive fouling and requires regular cleaning (Perry et al., 1988).

2.4.2.2. Open mode

Open mode refers to a situation in which the feed stream enters the shell or lumen and a portion of the feed either exits the shell or lumen while the other portion of the feed permeates through the membrane and exits through the lumen or shell in continuous stream, respectively (Bruining, 1989; Thakaran & Chau, 1986). The amount of feed that will permeate through the membrane is dependent on several factors, including the trans-membrane pressure, membrane permeability and feed velocity (Perry et al., 1988). Open mode is often favoured over closed mode as a lower development of fouling is encountered in the open mode operation.

2.4.3. Types of membrane bioreactors

MBRs may be classified as follows according to the function for which the membrane is being either utilised or located. Immobilised MBRs have shown huge potential, especially with the use of asymmetric membranes (graded porosity in the membrane structure) for the effective immobilisation of biofilms onto the membrane’s surface which, in turn, allows for (1) the transport of nutrients to the biomass immobilised on their external surface; (2) nutrients’ permeation gradient along the membrane; and (3) a shear-free environment for the production of value-added secondary metabolites (Godongwana, 2007; Leukes, 1999).
However, the application of these MBRs is limited to microorganisms with rhizoid growth that are able to penetrate this type of membrane structure to provide a stable attachment and is conventionally operated in the closed mode of membrane operation. Immersed and side stream MBRs are used for conventional biomass rejection, thus allowing for continued biomass utilisation (De Jager, 2009). In immersed and side stream MBRs, the membrane module is located in and separate from the bioreactor which is conventionally operated using either the closed or open mode of membrane operation, respectively. The disadvantages of side stream MBRs to immersed MBRs include the following: (1) they may be difficult to operate; (2) high energy requirements for the pump to maintain high pressure and volumetric flows; and (3) high capital cost. However, the separated system of bioreactor and membrane unit in side stream MBRs makes it easier to specifically optimise certain parameters for each unit. The latter is not possible in immersed MBRs (De Jager, 2009).

2.5. Cyanides and heavy metals in electroplating effluent

2.5.1. Background

CN-based electroplating operations are one of the most common types of electroplating processes. The electrolyte solution utilised in these operations generally comprises the following: (1) heavy metal CN complexes which contain the heavy metal(s) to be plated; (2) simple CN compounds to facilitate and improve anode corrosion or heavy metal CN complex decomposition, maintain a constant heavy metal ion level(s) and increases the conductivity in the bath to lesser degree; and (3) carbonates, phosphates and/or hydroxides to increase conductivity and buffer the pH in the bath to enhance throwing power and maintain an alkaline pH (Cushnie & CAI Resources Inc., 2009).

CNs are compounds/complexes that contain a CN functional group in which the carbon atom is attached to a nitrogen atom (−C≡N) while heavy metals is a loosely defined term used to describe chemical elements, such as certain transition metals, metalloids, lanthanides and actinides, which exhibit metallic properties (Nesbitt, 1996; Rao et al., 2010). However, there are various characteristics that define heavy metals including: (1) density; (2) atomic number/weight; and/or (3) chemical properties/toxicity. Although some heavy metals are toxic with low density, there are others which are not toxic and with a high density. The toxicity of heavy metals is commonly used to define heavy metals. The degree of toxicity may vary widely, depending on the metal's allotrope or oxidation state (Sud et al., 2008; Patil & Paknikar, 2000).
F-CN is the simplest form of CN and has two forms, namely, HCN and CN⁻ (Nesbitt, 1996). By definition, F-CN is released in aqueous solution by the dissolution and dissociation of CN compounds/complexes. The CN compounds/complexes commonly found in the electrolyte solution of a CN-based electroplating operation are defined as follows (Nsimba, 2009; Nesbitt, 1996):

- **Simple CNs**, such as KCN and NaCN, are ioniically bonded CN anions and alkali earth or alkali metals compounds that are electrically neutral and can exist in solid form. Simple CN compounds also dissociate into alkali earth or alkali metals and free cyanide when placed in aqueous solutions.

- **WAD-CNs**, such as tetracyanonickelate ([Ni(CN)₄]²⁻), tetracyanzincate ([Zn(CN)₄]²⁻) and tricyanocuprate ([Cu(CN)₃]²⁻), are heavy metal CN complexes which break down into F-CN and transition metal when in a weak acid aqueous solution (pH ≤ 4.5).

- **SAD CNs**, such as hecacyanoferrate (III) ([Fe(CN)₆]³⁻), hexacyanoferrate (III) ([Fe(CN)₆]⁴⁻) and tetracyanomercurate (I) ([Hg(CN)₄]²⁻), are heavy metal CN complexes, which are more stable than WAD-CNs and which break down into F-CN and transition metals when in a strong acid aqueous solution (pH ≤ 1).

For safety reasons it is advisable to keep CN solutions at a high pH to prevent the evolution of HCN as there is a direct relationship between the dissociation of HCN and pH (see Figure 2.5) (Nsimba, 2009; Nesbitt, 1996). HCN is a toxic and colourless gas with a distinctive almond smell at low concentrations only and slightly soluble in water. It readily dissociates into H⁺ and CN⁻ at high pH (Nesbitt, 1996).

![Figure 2.5: Relationship between HCN and pH (Nsimba, 2009; Nesbitt, 1996)](image)

The effluent derived from electroplating operations is mainly formed from the rinsing of the plated workpieces which, in turn, produces an effluent containing significant quantities of F-CN and heavy metal, including a lesser degree of WAD-CNs/SAD-CNs contaminants (Cushnie & CAI Resources Inc., 2009).
2.5.2. Conventional physical and chemical treatment methods

2.5.2.1. Alkaline chlorination

Alkaline chlorination is the most utilised and oldest technology used for the treatment of F-CN and WAD-CN. This method utilises oxidation using chlorine gas under alkaline conditions. The first step involves the formation of a CN chloride by the reaction of the CN and chlorine gas (Nsima, 2009; Nesbitt, 1996):

\[ CN^- + Cl_2 \rightarrow CNCl + Cl^- \]  

(Eq. 2.01)

The second stage involves the rapid alkaline based hydrolysis of the CN chloride to cyanate (Nsima, 2009; Nesbitt, 1996):

\[ ClCN^- + 2OH^- \rightarrow OCN^- + Cl^- + H_2O \]  

(Eq. 2.02)

The third stage involves the oxidation of the cyanate by chlorine gas to form carbonate and nitrogen gas (Nsima, 2009; Nesbitt, 1996):

\[ 2OCN^- + 6OH^- + 3Cl_2 \rightarrow 2HCO_3^- + 6Cl^- + 4OH^- + N_2 \]  

(Eq. 2.03)

Despite the fact that it is a very well-established process, with relatively low capital cost requirements and is both simple and versatile, the control over the quantity of chlorine gas that must be used in the system is restricted by fluctuations in CN concentration, pH, temperature, aeration and heavy metal content in the effluent and this may be problematic.

2.5.2.2. Copper catalysed hydrogen peroxide

Cu catalysed hydrogen peroxide is the second most utilised process and involves an oxidative reaction between hydrogen peroxide, in the presence of Cu, with F-CN, WAD-CN and/or cyanates (Nesbitt, 1996). The reaction mechanism for the oxidation of F-CN, WAD-CN and cyanates are depicted in Eqs. 2.04, 2.05 and 2.06, respectively (Khodadadi et al., 2005):

\[ CN^- + H_2O_2 \xrightarrow{Cu} CNO^- + H_2O \]  

(Eq. 2.04)

\[ M(CN)_4^{2-} + 4H_2O_2 + 2OH^- \xrightarrow{Cu} M(OH)_2 \downarrow + 4OCN^- + 4H_2O \]  

(Eq. 2.05)

\[ OCN^- + 2H_2O_2 \xrightarrow{Cu} CO_3^{2-} + NH_4^+ \]  

(Eq. 2.06)

where \( M \) represents metallic species such as Ni, Zn and Cu.
The Cu catalysed hydrogen peroxide treatment of F-CN, WAD-CN and/or cyanates containing effluent is also a very well established process as it is simple, the capital costs are low and it is possible to recover WAD-CN heavy metals. However, this process has high reagent costs and it cannot be applied to effluents containing high Fe complexed CNs as they react with Cu to form an insoluble complex.

2.5.2.3. Sulphur dioxide/air oxidation

Sulphur dioxide/air involves the oxidation of F-CN and WAD-CN using a gaseous mixture of sulphur dioxide and oxygen derived from air, in the presence of Cu, as depicted in Eqs. 2.07 and 2.08, respectively (Devuyst et al., 1989).

\[
CN^- + SO_2 + O_2 + H_2O \xrightarrow{Cu} OCN^- + H_2SO_4 \quad (\text{Eq. 2.07})
\]

\[
M(CN)_4^{2-} + SO_2 + 3O_2 + 2H_2O \xrightarrow{Cu} M(OH)_2 \downarrow + 4OCN^- + H_2SO_4 \quad (\text{Eq. 2.08})
\]

The cyanate produced requires further processing in order to remove it and this, in turn, results in increased reagent usage (Nesbitt, 1996). However, Fe CN complexes are reduced to a ferrous state (Eq. 2.09) and are then continuously precipitated as an insoluble metal ferrocyanide (Eq. 2.10) (Nesbitt, 1996):

\[
2Fe(CN)_6^{3-} + SO_2 + 2H_2O \xrightarrow{Cu} 2Fe(CN)_6^{4-} + 4H^+ + SO_4 \quad (\text{Eq. 2.09})
\]

\[
2M^{2+} + Fe(CN)_6^{4-} + H_2O \xrightarrow{Cu} M_2Fe(CN)_6 \cdot H_2O \downarrow \quad (\text{Eq. 2.10})
\]

where \(M\) represents metals such as Ni, Zn and Cu.

Lime or caustic soda is often used to neutralise the sulphuric acid that is produced in this process (Devuyst et al., 1989). Despite the fact that this is an established technology, the major drawback to this process is the high operational cost requirements.

2.5.2.4. Recovery using ion-exchange resin

Ion-exchange is an adsorption process that can adsorb heavy metals and CNs using anion and cation exchange resins, respectively (Nesbitt, 1996). However, the main drawback to this process is the selectivity of certain resins, competition of some species in the effluent and generates further waste from the acid/alkaline generation of the resin bed. Under normal circumstances, waste from the regeneration process requires further treatment.
2.5.3. Recent developments in the bioremediation of cyanides and heavy metals

2.5.3.1. Background

In recent years, research has focused on developing environmentally friendly bioremediation methods for hazardous contaminants, such as CNs and heavy metals. This is largely as a result of the fact that conventional chemical and physical treatment methods may be ineffective for certain CNs and heavy metals and produce further toxic by-products that require further treatment in order to meet municipal discharge standards. This, in turn, may involve high operative and capital costs. The use of biomaterials to treat effluent contaminated with CNs and heavy metals has also received considerable attention. This is as a result of the fact that these processes are cheap and possess unique bioremediation mechanisms and efficiencies that exceed those of the conventional physical and chemical treatment methods (Gupta et al., 2010; Wang & Chen, 2009). Biomaterials may be classified as: (1) inactive biomaterials or non-functional biomaterials such as agricultural residue and dead microbial biomass; and (2) active biomaterials or living biomaterials such as living microbial biomass.

2.5.3.2. Cyanide conversion

The catalytic/pseudo-catalytic conversion of CNs in effluent by biomaterials has shown significant potential as an environmentally friendly bioremediation method. However, these two concepts are often misunderstood as both of them increase the rate of reaction by lowering the energy required for the reaction to occur. Nevertheless, the main difference between a catalyst and a pseudo-catalyst is that the catalyst is not consumed by the reaction. The catalytic/pseudo-catalytic activity is the expression of how the rate of reaction of the substrate is increased by the presence of the catalyst/pseudo-catalyst. Temperature increases may result in an increased rate of reaction as the substrate has more free energy available, causing increased movement, collisions and rate of reaction. Similarly, if the substrate is susceptible to H⁺ or OH⁻ cleavage, the lower or higher the pH, the higher the concentration of H⁺ or OH⁻, respectively and, thus, the higher the rate of reaction. The larger the reactive surface interface between the catalyst/pseudo-catalyst, the greater the increased rate of reaction as a result of the higher availability of reactive sites. Promoters may also be employed to improve/maintain the rate of reaction by limiting the effect of negative parameters. However, inhibitors may cause a reduction in the rate of reaction or complete deactivation of the catalyst/pseudo-catalyst by binding to the active sites of the catalyst/pseudo-catalyst. Aging of the catalyst/pseudo-catalyst inevitably causes a reduction in its activity as a result of long-term usage and/or by changes in the morphology of active sites (Fogler, 2006).
Biomaterials that contain free hydroxyl functional groups, such as agricultural residues, may pseudo-catalytically convert CNs, a technique which is largely unreported in the literature reviewed. The free hydroxyl group may behave as a weak acid and deprotonate to produce an alkoxide in the presence of a strong base, such as CN. This proton will hydrolytically convert the carbon-nitrogen triple bond in the CN and initiate its decomposition into ammonia/NH$_4^+$ and carboxylic acid/carboxylate, depending on pH. One of the key factors that may significantly affect the conversion of CNs is species which would, in turn, block the free hydroxyl functional group, such as heavy metals that have an affinity for the free hydroxyl group. Alternatively, there are various enzymatic pathways in which enzymes catalytically convert CNs into organic/inorganic molecules. The particular enzymatic pathways and enzymes associated with the catalytic conversion of CNs include the following (Gupta et al., 2010; Rao et al., 2010):

- **Hydrolytic conversions** involve either the enzymatic conversion of CN by Class 1 enzymes: (1) cyanide hydratase; (2) nitrile hydratase; or (3) thiocyanate hydrolase, to form formamide and/or Class 2 enzymes: (1) nitrilase; or (2) cyanidase, to form ammonia/NH$_4^+$ and carboxylic acid/carboxylate, depending on the pH.
- **Oxidative conversion** involves the oxygenolytic conversion of CNs by CN monooxygenase and cyanase or CN dioxygenase to carbon dioxide and ammonia/NH$_4^+$, depending on the pH.
- **Reductive conversion** is under anaerobic conditions in which the enzymatic conversion of CNs by the nitrogenase produces methane and ammonia/NH$_4^+$, depending on the pH.
- **Substitution/transfer conversions** are as a result of the enzymatic assimilation of CNs by the enzymes rhodanese, mercaptopyruvate or sulfurtransferase, using CN as a nutrient source, thus preventing CN toxicity in the microorganisms producing the enzymes.
- **Synthase conversions** are another enzyme-catalysed assimilation processes for CNs by the enzymes β-cyanoalanine or γ-cyano-α-aminobutyric acid synthase and which synthesise amino acids β-cyanoalanine and γ-cyano-α-aminobutyric acid, respectively, by the utilisation of amino acid residues as the precursors that react with CNs.

However, enzymatic catalysis is a complicated process and the enzymatic conversion of CNs may be significantly affected by the mechanism involved, initial CN concentration, microbial tolerance to CN, temperature, pH and the presence of by-products from CN conversion and various heavy metal ions, including the fluctuations of these parameters (Gupta et al., 2010). Similarly, enzymes operate optimally at specific pH and temperature conditions and any variations in these parameters may result in the inactivation of the enzyme (Fogler, 2006).
2.5.3.3. Heavy metal uptake

The use of biomaterials to removal heavy metals from effluent has shown significant potential as an environmentally friendly bioremediation method. The heavy metal removal mechanism is dependent on the activity of the biomaterial. Inactive biomaterials act as ion-exchangers between heavy metals and the functional groups present in both the inactive biomaterial (biosorption) and the active biomaterials in which the heavy metals are biosorbed and assimilated (bioaccumulation) by ionic interactions and metabolic processes, respectively (Eccles, 2000). Functional groups associated with biomaterials have specific heavy metal affinities (see Table 2.1).

Table 2.1: Functional groups and their heavy metal affinity (Eccles, 2000)

<table>
<thead>
<tr>
<th>Cell wall functional group</th>
<th>Metal affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxyl</td>
<td>Ca, Mg, Cu &amp; Zn</td>
</tr>
<tr>
<td>Imidazole</td>
<td>Cu &amp; Pb</td>
</tr>
<tr>
<td>Sulphhydrol</td>
<td>Zn</td>
</tr>
<tr>
<td>Amino</td>
<td>Co, Ni &amp; Cu</td>
</tr>
<tr>
<td>Phosphate</td>
<td>Ca, Mg, Fe &amp; U</td>
</tr>
<tr>
<td>Sulphate</td>
<td>Ba, Ca &amp; Sr</td>
</tr>
<tr>
<td>Thioether</td>
<td>Cu</td>
</tr>
<tr>
<td>Amide</td>
<td>Cu, Co, Ni &amp; Fe</td>
</tr>
<tr>
<td>Hydroxyl</td>
<td>Ca, Pb, Cu, Sr, Ba, Ni, Co &amp; Zn</td>
</tr>
</tbody>
</table>

The bioaccumulation of heavy metals for metabolic processes may offer a unique way in which heavy metal can be removed and which is impossible in inactive biomaterials and using conventional treatment methods. However, the facilitation of such a process using living microorganisms requires that the organisms possess mechanisms to overcome the detrimental effects of the heavy metals, but can also be used to remediate other pollutants, such as CNs (Eccles, 2000). Nevertheless, inactive biomaterials offer added advantages over active biomaterials as their heavy metal removal process is: (1) largely independent of toxicity limitations; (2) requires no growth media and nutrients; (3) adsorbed metals may be removed and the biomaterials may be reused after regeneration; (4) a large quantity of these biomaterials may be easily obtained; (5) pre-treatment of biomaterials may enhance heavy metal biosorption; (6) the process is simpler as compared to ion-exchange; (7) disposal of unused and/or surplus biomass is not problematic as the unused or surplus biomass may be used for heavy metal uptake; and (8) their near infinite supply and shelf-life (Eccles, 2000). However, the surface characteristics of active biomaterials may change with the biomaterial age.
In addition, the composition or nature of the growth media and various organic materials, such as agricultural residues, may have different functional groups, thus imparting different properties onto the biomaterial (Wan Hgah & Hanafiah, 2008; Eccles, 2000). The key factors affecting the uptake of heavy metals by biomaterials include pH, temperature, competing heavy metal species, types of functional groups present in the biomaterial and heavy metal speciation. Despite the fact that all these factors are equally important with regards to the removal of heavy metals, pH does influence the mechanism of the protonation and deprotonating of the functional groups and is, thus, a far more dominant factor than the other factors (Igwe et al., 2011; Eccles, 2000).

For example, in highly acidic conditions, pH ≤ 3, the heavy metal removal efficiency by the biomaterial will be low as the heavy metal has to complete with hydrogen ions. This is as a result of the fact that most functional groups already contain an exchangeable hydrogen ion and this, in turn, will result in minimal ion exchange with the heavy metals in the effluent (Igwe et al., 2011; Eccles, 2000). However, as the pH is increased to mildly acidic conditions, 3 < pH < 6, the competition between the heavy metal and hydrogen ion increases the dissociation of the hydrogen ion from the functional group for improved ion-exchange with heavy metals.

At pH near neutral, 6 < pH < 8, the heavy metal removal efficiency decreases as a result of the equilibrated competition between the heavy metals and hydrogen ions (Igwe et al., 2011). As the pH is increased to a higher pH, pH > 8, the ion-exchange between the heavy metal and hydrogen ion in the functional groups increases significantly as a result of the rapid decline in competition between the heavy metals and hydrogen ions, thus increases the dissociation of the hydrogen ion from the functional group. This, in turn, results in a change in the speciation of the heavy metal from being a hydrated cation to a hydroxyl heavy metal species which provides improved heavy metal binding efficiency (Eccles, 2000).

Similarly, the effect of temperature may have either a positive or a negative effect on the uptake of heavy metals. As the temperature increases, the binding of heavy metal specie(s) on functional group(s) increases as a result of the increasing kinetic energy of the heavy metal specie(s), thus resulting in a more spontaneous ion-exchange interaction force with the functional group(s), up to a critical temperature. The critical temperature is the point at which the ionic binding energy between the heavy metal specie(s) and functional group(s) is at its maximum and its most stable. Any increase in temperature beyond this point results in higher kinetic energy for the heavy metal specie(s) and a weaker and unstable ion-exchange interaction force with the functional group(s) (Murthy et al., 2012; Horsfall & Spiff, 2005).
2.5.4. Application of *Aspergillus* section *Nigri* and agricultural residues

2.5.4.1. Background

The black *Aspergilli* (*Aspergillus* section *Nigri*) species, an aerobic filamentous fungi often derived from soil, has shown potential in both biotechnology and in food and medical mycology. The characteristics of these species which cause agricultural products to spoil have recently shown to be beneficial as a result of their potential to hydrolyse the carbohydrates (see Figure 2.6). This hydrolysis of agricultural residues may be used to produce a variety of industrially important extracellular enzymes, such as cellulase, amylase, xylanase, pectinase, elastase, and organic acids, for example, citric and galacturonic acid (Nigam & Pandey, 2009; Li *et al*., 2008; Papagianni, 2007; Mitchell *et al*., 2006).

![Carbohydrates](image)

**Figure 2.6: Classification of carbohydrates**

*Aspergillus* spp. also have hydrolytic nitrilase and cyanidase enzymes to convert CNs to ammonia/NH$_4^+$ and carboxylic acid/carboxylate, depending on pH, and then metabolise these products as nitrogen and carbon sources, respectively (Gupta *et al*., 2010; Rao *et al*., 2010). These enzymes have shown to have a higher activity and can convert various CNs, as compared to similar enzymes that are derived from bacteria (Rao *et al*., 2010). The conversion of CNs by these enzymes is facilitated by core amino acids: (1) glutamate; (2) cysteine; and (3) lysine (see Figure 2.7). Glutamate deprotonates the thiol group of cysteine which enables the nucleophilic attack of the cysteine on the carbon and the subsequent protonation of the nitrogen in the CN molecule. A water molecule is deprotonated by lysine which then launches a nucleophilic attack on the carbon in the CN molecule to form a tetrahedral intermediate. This intermediate, spontaneously broken-down via cleavage of the C-N bond producing an acylenzyme and ammonia. A second water molecule is deprotonated by glutamate, which then launches a nucleophilic attack on the thioester in the CN molecule to form a second tetrahedral intermediate. This intermediate then spontaneously decomposes to produce the carboxylic acid with release of the glutamate and cysteine (Thuku, 2006; Stevenson *et al*., 1992). As a result of the alkaline conditions, the carboxylic acid is deprotonated to form a carboxylate and this proton then protonates the ammonia to form NH$_4^+$. 
Figure 2.7: Enzymatic conversion of CN using nitrilase or cyanidase
In addition, *Aspergillus* spp. constitute an alternative respiration pathway which makes them particularly tolerant to the high CN concentrations which would cause lethal respiration inhibition in other microorganisms (Hattori, 2008). However, despite the potential of agricultural residues as a suitable feedstock for the production of value-added products, there are limited studies available which have shown their potential as a feedstock in a bioreactor for the bioremediation of CNs and heavy metals.

Similarly, the potential of agricultural residues that contain significant quantities of free hydroxyl functional groups to act as a pseudo-catalyst for the conversion of CNs (see Figure 2.8) has not been reported in literature. The free hydroxyl functional group behaves as a weak acid, especially at increasing alkaline pH, and deprotonates to produce an alkoxide in the presence of a strong base, such as CNs. This proton subsequently protonates the nitrogen in the CN molecule, thus facilitating the destabilisation of the carbon-nitrogen triple bond in the CN and initiating its decomposition. A hydroxide ion then launches a nucleophilic attack on the carbon in the CN molecule to produce an alkoxide intermediate. A water molecule then launches a nucleophilic attack on the alkoxide intermediate to initiate its decomposition and produce a carboxylic acid and ammonia. As a result of the alkaline conditions, the carboxylic acid is deprotonated to form a carboxylate and this proton then protonates the ammonia to form NH$_4^+$.

![Figure 2.8: Pseudo-catalytic conversion of CN by the free hydroxyl functional group](image-url)
The potential of agricultural residues that contain significant quantities of free hydroxyl functional groups may be positive when these agricultural residues are used with *Aspergillus* spp. for the bioremediation of the CNs and heavy metals in effluent. However, this concept is novel in approach and further research is required.

2.5.4.2. Design methodology for a membrane bioreactor system for the continuous bioremediation of cyanides and heavy metals in effluent

The recent increase in the bioremediation technologies of CNs and heavy metals has resulted from the potential environmental friendliness and sustainability that they offer. Similarly, there is a need to develop the market further by proposing agricultural residues as primary feedstock for active microbial processes. However, there have been limited studies conducted utilising this method, despite the fact that conventional treatment methods are not environmentally friendly and are characterised by high capital and reagent costs. This is especially evident in the treatment methods of effluent which consists of both CNs and heavy metals (Gupta *et al*., 2010; Patil & Paknikar, 2000; Nesbitt, 1996). The development and design of an immersed MBR system for the continuous bioremediation of the CNs and heavy metals in effluent would be advantageous because of the: (1) containment of catalyst and pseudo-catalyst in the MBRs using ultrafiltration (UF) membranes; (2) lower energy requirements; and (3) uncomplicated characteristics of this type of MBR. In addition, the challenges associated with membrane fouling may be overcome by regular back pulsing and CN volatilisation may be reduced by the design of a contained immersed MBR design to offer a safe operational bioreactor unit (Santos *et al*., 2013). Nevertheless, active biomaterial bioremediation processes may exhibit unique contaminant conversion mechanisms and the subsequent metabolism of possible by-products from treated effluent which is not possible with inactive biomaterials. These bioremediation processes tend to be more sensitive to contaminant fluctuations and concentrations which could render the process redundant (Santos *et al*., 2013). It is also essential that the use of agricultural residue as nutrient supplements for a bioremediation process be evaluated and also the reduction of external chemical usage be investigated in the interests of a cost effective and environmentally sound bioremediation method. A two-stage immersed MBR system utilising a primary inactive bioremediation stage to reduce fluctuations and remove contaminants in the effluent and a secondary active bioremediation stage to further convert CN and remove heavy metals and remediate residual contaminants in the effluent would combat this issue. In addition, this hybrid system would, theoretically, provide conditions which would be conducive for a suitable bioremediation process, although there have been limited studies which have used such a system for the combined bioremediation of CNs and heavy metals in effluent.
CHAPTER 3
MATERIALS AND METHODS
CHAPTER 3
MATERIALS AND METHODS

3.1. Introduction

This chapter describes the materials and methods used during experimentation. The chapter also discusses some of the analytical tools which were used to present and/or assess the results obtained. All the experiments and analyses were conducted in duplicate to ensure that they were reproducible.

3.2. Experimental design

Central composite design (CCD) is one of the most widely used approaches for determining an optimum or to analyse a response to changes in the variables for various processes. This design utilises the response covered in an experimental design which has been developed, such that the monitored output variables may be analysed efficiently. Response Surface Methodology (RSM) is a combination of mathematical and statistical techniques that utilises quantitative data to develop and solve multivariate equations in order to model the effect of several changing variables on the response of the system under evaluation. Accordingly, a CCD is a major component of the statistical design of experiments, with RSM being the instrument used for analysing the response obtained and which enables the representation of the independent process parameters to be expressed as:

\[ Y_i = f(X_1, X_2, X_3, \ldots, X_n) \pm \varepsilon \quad \text{(Eq. 3.01)} \]

where \( Y_i \) is the response, \( X_1, X_2, X_3, \ldots, X_n \) are the coded independent variables and \( \varepsilon \) is the experimental error.

However, this cannot be done using a function in Eq. 3.01, since the form of \( f(X) \) is unknown and may be complex. Therefore, RSM utilises an approximation method for \( f(X) \) by trying to fit a lower ordered polynomial to model the effect of the changing independent variables on the response of the system. If the function can be described by a linear function of independent variables, then the response may be expressed as follows:

\[ Y_i = b_0 + \sum_{i=1}^{k} b_{1(i)} X_i \pm \varepsilon \quad \text{(Eq. 3.02)} \]

Where \( b_{1(i)} \) are the linear coefficients and \( b_0 \) is a constant.
However, in most cases, the response to the changing variables results in a curvature in the response and, thus, a higher order polynomial has to be used, such as a quadratic polynomial:

\[
Y_i = b_0 + \sum_{i=1}^{k} b_{1(i)}X_i + \sum_{i=1}^{k} b_{2(i)}X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} b_3X_iX_j \pm \epsilon \tag{Eq. 3.03}
\]

where \( b_{2(i)} \) is quadratic coefficient and \( b_3 \) is the interactive coefficient.

Once an appropriate polynomial has been fitted to the response, RSM will enable the analysis of certain responses at certain independent variable ranges or in order to optimise the response. Temperature \((X_1)\) and pH \((X_2)\) were selected as the critical independent variables since both of these variables affect the response, i.e. CN and heavy metal bioremediation, with a varying degree of magnitude. Design Expert V8.0 (Stat Ease, USA) was used to generate an experimental design (see Table 3.1). Furthermore, the pH and temperature ranges selected were deemed appropriate to support a bioreactor for electroplating effluent bioremediation.

**Table 3.1: Experimental ranges for pH and temperature**

<table>
<thead>
<tr>
<th>Run</th>
<th>Temperature (˚C)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35.00</td>
<td>9.50</td>
</tr>
<tr>
<td>2</td>
<td>35.00</td>
<td>12.00</td>
</tr>
<tr>
<td>3</td>
<td>50.00</td>
<td>9.50</td>
</tr>
<tr>
<td>4</td>
<td>24.39</td>
<td>11.27</td>
</tr>
<tr>
<td>5</td>
<td>45.61</td>
<td>7.73</td>
</tr>
<tr>
<td>6</td>
<td>20.00</td>
<td>9.50</td>
</tr>
<tr>
<td>7</td>
<td>35.00</td>
<td>9.50</td>
</tr>
<tr>
<td>8</td>
<td>24.39</td>
<td>7.73</td>
</tr>
<tr>
<td>9</td>
<td>35.00</td>
<td>7.00</td>
</tr>
<tr>
<td>10</td>
<td>35.00</td>
<td>9.50</td>
</tr>
<tr>
<td>11</td>
<td>35.00</td>
<td>9.50</td>
</tr>
<tr>
<td>12</td>
<td>35.00</td>
<td>9.50</td>
</tr>
<tr>
<td>13</td>
<td>45.61</td>
<td>11.27</td>
</tr>
</tbody>
</table>

Eqs. 3.04 and 3.05 are the linear and quadratic functions which were best suited to model the responses, respectively. These functions, in terms of two independent variables, may be expressed as follows:

\[
Y_i = b_0 + b_{1(1)}X_1 + b_{2(2)}X_2 \pm \epsilon \tag{Eq. 3.04}
\]

\[
Y_i = b_0 + b_{1(1)}X_1 + b_{1(2)}X_2 + b_{2(2)}X_1^2 + b_{2(2)}X_2^2 + b_3X_1X_2 \pm \epsilon \tag{Eq. 3.05}
\]
3.3. Experimental material preparation

3.3.1. Solid media preparation

The following solid media were prepared and used at various stages during the research:

- PA solution was prepared by adding 10 g Citrus pectin and 20 g Agar-agar to a 1 L Schott bottle and a 1 L solution made using distilled water.
- PDA solution was prepared by adding 39 g PDA powder to a 1 L Schott bottle and a 1 L solution made using distilled water.
- Malt extract agar (MEA) was prepared by adding 50 g MEA powder to a 1 L Schott bottle and a 1 L solution made using distilled water.
- Czapek yeast agar (CYA) was prepared by adding 51 g CYA powder to a 1 L Schott bottle and a 1 L solution made using distilled water.

The agar solution was stirred using a magnetic stirrer for 5 minutes and the agar solution was then autoclaved at a temperature of 121 °C for 20 minutes and then left to cool to a workable temperature (± 50 °C) under a sterile conditions.

Antibiotic versions of these agar solutions may be made by pipetting 2 mL of Penicillin-Streptomycin antibiotic solution (10,000 units/L penicillin and 10 mg streptomycin/mL) into the agar solution once the agar solution has cooled to a workable temperature (± 50 °C). The agar solution must be slowly swirled for 2 minutes to disperse the antibiotic.

F-CN versions of these agar solutions can be made by making an agar solution using double the recommended amount of the agar constituent(s). The agar solution was then autoclaved at a temperature of 121 °C for 20 minutes, left to cool to ± 50 °C and maintained at this temperature using a magnetic stirrer under sterile conditions. A series of 80 mL F-CN agar solutions were prepared in a sterile 100 mL Schott bottle using a 1000 mg F-CN/L solution, 2.5 g KCN/L distilled water, and sterile distilled water. The Schott bottle’s contents were stirred while the diluted F-CN solution was added slowly to efficiently disperse the F-CN solution while the solution was being stirred using a magnetic stirrer.

The agar solution was poured into Petri dishes using a flaming technique to prevent transferred contamination. In other words, the agar solution bottle was flamed using a Bunsen burner both before and after the agar solution had been poured into the Petri dishes. The Petri dishes were filled with agar solution until a thickness of ± 0.5 cm, left to cool and set, that is, until the agar was characterised by a stiff and jelly like consistency.
3.3.2. Microorganism isolation and inoculum preparation

Swabs were taken at various points along a municipal discharge drain at the electroplating facility. PA plates were inoculated using the swabs, parafilmed and incubated for 5 days at 37 °C to isolate microorganisms with the ability to grow solely on materials containing pectin. The colonies on the PA plates were transferred onto the antibiotic PDA plates and incubated for a further 5 days at 37 °C. Subsequently, conidia from sporulated isolates were transferred to PDA plates and incubated for an additional 5 days at 37 °C to obtain pure cultures. After incubation, a spore-mycelia suspension was prepared by pipetting 5 mL sterile distilled water onto the PDA plates and gently swirling the washing solution so as to immobilise the conidia. Subsequently, the washing solution was transferred into a sterile 100 mL Schott bottle under sterile conditions. This procedure was repeated for all the PDA plates. In order to separate spores from mycelia, sterilised glass-wool was placed in sterile 60 mL syringes, the solution containing spores and mycelia was poured into the top-end of the syringes containing the sterile glass-wool and the plunger was pushed down to separate the spores from the mycelium by entrapping the mycelia on the glass-wool, while the recovered spore-containing solution was transferred into another sterile 100 mL Schott bottle. The spore solution was stored at 4 °C prior to being used in the experiments. A series of dilutions was performed using the spore solution and sterile distilled water to quantify the spore concentration. The spore concentration and absorbance of the each of the spore dilutions was determined in duplicate using both a direct count system in a Marienfeld Neubauer cell-counter and a Nikon Eclipse E2000, phase contrast 1 and 100 × magnification. In addition, a Jenway 6715 UV/Visible spectrophotometer was used at 750 nm using sterile distilled water as a blank to develop an absorbance-spore concentration graph (Torrado et al., 2011). A calibration graph for the spore concentration was determined by plotting absorbance versus the spore concentration (see Figure 3.1).

![Figure 3.1: Calibration curve for spore concentration](image)

Absorbance = 0.0737 [Spore] × 10^6 - 0.0354
R² = 0.9996
3.3.3. Drying agricultural residue

The raw agricultural residues, namely, *C. sinensis* pomace, *M. domestica* pomace, *Z. mays* cob and *S. tuberosum* peel, were washed twice in distilled water to remove any free debris. This washed agricultural residue was then dried for 72 hours at 80 °C and then ground into a fine powder (≥ 100 µm) using a grinder (Bosch MKM 7000).

3.3.4. Preparation of unhydrolysed, hydrolysed and benzylated agricultural residue

To prepare the unhydrolysed agricultural residue, 60 g of the ground residue was added to a 1 L Schott bottle and a 1 L solution was made using sterile distilled water. The solution was stirred for 5 minutes using a magnetic stirrer and then filtered through a No 1 Whatman filter paper using a Büchner funnel under vacuum. The filter cake was dried at 80 °C for 24 hours and then ground to a fine powder (≥ 100 µm) using a grinder (Bosch MKM 7000) and stored at room temperature. To prepare the hydrolysed agricultural residue, 60 g of the ground residue, 800 mL distilled water and 5 mL H$_2$SO$_4$ (98%) were added together and mixed in a 2 L Schott bottle and a 1 L solution was made using distilled water. The solution was autoclaved at 116 °C for 13 minutes and then cooled to room temperature (Talebnia *et al.*, 2008). The pH was adjusted to 4.5 with 1 M NaOH, stirred for 5 minutes using a magnetic stirrer and then filtered through a No 1 Whatman filter paper using a Büchner funnel under vacuum. The filter cake was dried at 80 °C for 24 hours and then ground to a fine powder (≥ 100 µm) using a grinder (Bosch MKM 7000) and stored at room temperature. The agricultural residue extract was transferred into a 2 L Schott bottle, a 2 L solution was made using sterile distilled water and stored at 4 °C and then used as a media for isolate supplementation. To prepare the benzylated agricultural residue, 16 g of KOH was dissolved in 32 mL distilled water in 250 mL round bottom flasks. A mass of 8 g of either hydrolysed or unhydrolysed residue was added to the flask and the mixture was mixed by swirling the flask. The flask was placed on a Labmaster isopad (LMUL/ER/500ML) heater and fitted with a reflux condenser under a fume hood. The mixture was heated to a gentle boil, after which 12 mL bromobenzene was added drop-wise through the condenser to the boiling solution over a period of 10 minutes. After the bromobenzene had been added, the mixture was left to reflux for 10 minutes. The mixture was then transferred to a 250 mL beaker and left to cool to room temperature. The pH of the mixture was adjusted to 4.5 using HCl (32%) and then cooled in an ice bath. The mixture was filtered through a No 1 Whatman filter paper using a Büchner funnel under vacuum. The recovered filter cake was dried for 24 hours at room temperature and then ground to a fine powder (≥ 100 µm) using a grinder (Bosch MKM 7000) and stored in a desiccating chamber at room temperature.
3.3.5. Active and inactive *Aspergillus awamori* biomass

Active *A. awamori* biomass was produced by inoculating 200 mL agricultural residue extract with a 1 mL inoculum solution (20 × 10⁶ spores) in a 500 mL flask and the flasks were incubated in a rotary shaker at 35 °C and 150 rpm for 24 hours. After the 24 hours incubation, 50 mL of a phosphate buffer (pH 9.5) was added to each flask to increase the pH of the media and the incubation was continued at 35 °C and 150 rpm for 24 hours. After the 48 hours of incubation, active biomass was obtained by filtering the mixture through a No 1 Whatman filter paper using a Büchner funnel under vacuum. The biomass was then washed using sterile distilled water. A mass of 1.2 g of wet *A. awamori* biomass, equivalent to 0.05 g dry *A. awamori* biomass, was weighed and used for the heavy metal uptake study. Inactive *A. awamori* biomass was grown and harvested following a similar procedure to that used for the active *A. awamori* biomass. After filtration, in order inactivate the biomass; it was autoclaved at 121 ºC for 15 minutes.

3.4. Experimental procedures

3.4.1. Electroplating effluent collection and sampling

Effluent samples from an electroplating facility located in the Western Cape, South Africa, were collected in 25 L polypropylene containers from the municipal discharge point and stored at 4 °C.

3.4.2. Free cyanide tolerance of the isolate

The F-CN PDA plates were inoculated using the microorganism which had been isolated as described in section 3.3.2 and the plates were sealed with parafilm and incubated at 37 °C for 5 days.

3.4.3. Identification of the isolate

Morphological studies were conducted by preparing, inoculating and incubating MEA and CYA plates at 26 °C for 7 days. The fruiting bodies on the plates were mounted in lactic acid before they were observed using a Nikon Eclipse E2000 at 100 × magnification. Molecular identification was performed to confirm the identification of the isolate. This was done by means of DNA extraction from pure colonies using the ZR Fungal/Bacterial DNA Kit (Zymo Research, California, USA). The subsequent Polymerase Chain Reaction (PCR) of the ITS1–5.8S–ITS2, β-tubulin and calmodulin gene regions were prepared with primers ITS1 and ITS4, Bt2a and Bt2b and CL1 and CL2A, respectively (Santos et al., 2013).
Sequencing reactions of the PCR products were set up using a Big Dye terminator cycle sequencing premix kit (Applied Biosystems, CA) and were analysed using an ABI PRISM 310 genetic analyser. The sequences were compared to those of a recent study by Varga et al. (2011) for the type of strains in this section. Datasets were aligned in Se-Al. Sequence analysis was carried out in PAUP* v4.0b10, using the BioNJ option for calculating a single tree for each dataset. The confidence in nodes was calculated using a bootstrap analysis of a 1000 replicates.

3.4.4. Suitability of agricultural residues

A 100 mL inoculum solution was made up by adding 10 mL agricultural residue extract and 1 mL spore solution (10 × 10⁶ spores) to a 100 mL Schott bottle. A 100 mL solution was then made using sterile distilled water. A volume of 1 mL of the inoculum solution was added to a 1.5 mL Eppendorf tube and incubated in a rotary shaker at 35 °C at 150 rpm for 24 hours. The inactive or active incubated inoculum solution or 0.05 g unhydrolysed or hydrolysed agricultural residue and 50 mL of the synthetically prepared electroplating solution, which had an average T-CN, Ni, Zn, and Cu concentrations of those determined for the electroplating effluent, were added to a 100 mL Schott flask. The flasks were incubated in a rotary shaker at 150 rpm and at 37 °C for 48 hours.

3.4.5. Free cyanide conversion by *Citrus sinensis* pomace

A mass of 0.05 g unhydrolysed or hydrolysed *C. sinensis* pomace was weighed and added to 50 mL (100 mg/L) F-CN solution in a 100 mL Schott flask. The (100 mg/L) F-CN solution was prepared by adding 0.250 g KCN and 800 mL phosphate buffer (pH values as stipulated in Table 3.1) to a 1 L Schott flask. A 1 L solution was made using sterile distilled water. The flasks were incubated in a rotary shaker at 150 rpm and at the required temperature (as stipulated in Table 3.1) for 48 hours.

3.4.6. Free cyanide conversion by *Aspergillus awamori* biomass

The preparation of the incubated inoculum solution followed the same procedure as discussed in section 3.4.4. The incubated inoculum solution and 50 mL (100 mg/L) F-CN media were added to a 100 mL Schott flask. The (100 mg/L) F-CN media was prepared by adding 0.250 g of KCN, 10 mL selected agricultural residue extract and 800 mL phosphate buffer (pH values stipulated in Table 3.1) to a 1 L Scott bottle. A 1 L solution was made using sterile distilled water. The flasks were incubated in a rotary shaker at 150 rpm and at the required temperature (as stipulated in Table 3.1) for 48 hours.
3.4.7. Heavy metal uptake by biomaterials

A mass of 0.05 g unhydrolysed or hydrolysed agricultural residue or 1.2 g of wet active or inactive A. awamori biomass, equivalent to 0.05 g dry A. awamori biomass, was weighed out and the weighed biomaterial and 50 mL (10 mg/L) heavy metal media were added to a 100 mL Schott flask. The (10 mg/L) heavy metal media was prepared by adding 0.050 g Ni(NO$_3$)$_2$.6H$_2$O, 0.037 g Cu(NO$_3$)$_2$.2.5H$_2$O, 0.040 g Zn(NO$_3$)$_2$.4H$_2$O, 10 mL selected agricultural residue extract and 800 mL phosphate buffer (pH values stipulated in Table 3.1) to a 1 L Schott flask. A 1 L solution was made using sterile distilled water. The flasks were incubated in a rotary shaker at 150 rpm and at the required temperature (as stipulated in Table 3.1) for 2 hours until equilibrium was reached (Li et al., 2008).

3.4.8. Pseudo-catalyst inhibition and deactivation of Citrus sinensis pomace

A mass of 0.05 g hydrolysed or benzylated agricultural residue was weighed out and the weighed residue, 50 mL (10 mg/L) heavy metal and (100 mg/L) F-CN or (100mg/L) F-CN solution were added to a 100 mL Schott flask. The (10 mg/L) heavy metal and (100 mg/L) F-CN solution was prepared by adding 0.050 g Ni(NO$_3$)$_2$.6H$_2$O, 0.037 g Cu(NO$_3$)$_2$.2.5H$_2$O, 0.040 g Zn(NO$_3$)$_2$.4H$_2$O, 0.250 g KCN and 800 mL to the phosphate buffer (pH 12) to a 1 L Schott bottle. A 1 L solution was made using distilled water. The (100 mg/L) F-CN solution was prepared by adding 0.25 g KCN and 800 mL to the phosphate buffer (pH 12) to a 1 L Schott bottle and a 1 L solution was made using distilled water. The flasks were incubated in a rotary shaker at 150 rpm and 40 °C for 48 hours.

3.4.9. Two-stage membrane bioreactor system for the continuous bioremediation of electroplating effluent

3.4.9.1. System construction

Five two-stage immersed MBRs were constructed according to the schematic diagrams illustrated in Figure 3.2 and sterilised using an autoclave at 121 °C for 15 minutes. The constructed MBR experimental setups which had been constructed were operated at a temperature of 40 °C with the immersed MBRs and collection bottles being shaken at 150 rpm in a rotary shaker. Watson Marlow 520S and Watson Marlow 101 U/R peristaltic pumps were used to pump the electroplating effluent and C. sinensis extract to and through the experimental setup using silicone tubing (6 mm × 5 mm), respectively.
The use of 60 mL BT syringes, with Luer-Lock tips, was intended to reduce CN volatilisation in the system. Asymmetric aluminium oxide ceramic capillary UF membranes used in the construction of the immersed MBRs were produced and supplied by Hyflux CEPAration BV (Netherlands) (see Table 3.2 for specifications).

Table 3.2: Asymmetric aluminium oxide capillary UF membrane specifications (De Jager, 2009)

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer diameter (m)</td>
<td>0.0028</td>
</tr>
<tr>
<td>Inner diameter (m)</td>
<td>0.0018</td>
</tr>
<tr>
<td>Wall thickness (m)</td>
<td>0.0005</td>
</tr>
<tr>
<td>Burst pressure (Pa)</td>
<td>5.0x10^6</td>
</tr>
<tr>
<td>Maximum temperature (˚C)</td>
<td>1000</td>
</tr>
<tr>
<td>Permeability (m/Pa.s)</td>
<td>6.95x10^-10</td>
</tr>
</tbody>
</table>

The ends of the membranes were sealed with Pratley Quickset Clear epoxy after sterilisation to form a closed mode of membrane module operation. The collection bottles and immersed MBRs were constructed according to the schematic diagrams presented in Figures 3.3 and 3.4 respectively.
Figure 3.3: Collection bottle (i) assembly and (ii) assembled schematic diagrams
Figure 3.4: Immersed MBR (i) assembly and (ii) assembled schematic diagrams
3.4.9.2. System start-up and operation

The preparation of the incubated inoculum solution followed the same procedure as discussed in section 3.4.4. An incubated inoculum and 50 mL diluted *C. sinensis* pomace extract solution were added to each of the active MBRs and the solution was incubated in a rotary shaker at 150 rpm and 40 °C for 48 hours. The 50 mL diluted *C. sinensis* pomace extract solution was made by adding 0.5 mL *C. sinensis* pomace extract and 49.5 mL sterile distilled water to a 100 mL Schott bottle. After incubation, 100 mL of the electroplating effluent and 50 mL of the diluted electroplating effluent solution were added to the inactive and active MBRs, respectively. The diluted electroplating effluent solution was made by adding 1 mL electroplating effluent and 49 mL sterile distilled water to a 100 mL Schott bottle. A mass of 11.11 g hydrolysed *C. sinensis* pomace was weighed out, equivalent to 10.00 g after being re-suspended in a solution, filtered and dried, and the weighed residue was added to the inactive MBR. The feed bottle was filled with electroplating effluent. The electroplating effluent feed was then pumped through the inactive MBR at a rate of 10 mL/hour using a Watson Marlow 520S peristaltic pump for 24 hours.

After 24 hours, the active MBRs were started by pumping intermediate effluent to the active MBR at 10.01 mL/hour using another Watson Marlow 520S peristaltic pump and supplying *C. sinensis* pomace extract to the intermediate collection bottle at a rate of 0.001 mL/hour, using a Watson Marlow 101 U/R peristaltic pump. Samples from the feed, intermediate and product collection bottles were then drawn simultaneously. The system was operated for 12 days and samples were withdrawn from the feed, intermediate and product collection bottles every 24 hours. After sampling, the feed collection bottle was filled with the electroplating effluent which had been collected and the product collection bottles were emptied. The electroplating effluent was collected every 12 hours and was arranged and administered as feed in fluctuating CN concentrations to evaluate the sensitivity of the process.

After every 4 days of continuous operation, the hydrolysed *C. sinensis* pomace was regenerated in each of the inactive MBRs and one of the experimental setups was deconstructed to quantify the mass of *A. awamori* biomass and hydrolysed *C. sinensis* pomace (see section 3.5.1.). The regeneration was performed by stopping the operation of the inactive MBRs and filtering and washing the suspension with 20 mL sterile distilled water through a No 1 Whatman filter paper using a Büchner funnel under vacuum. The effluent filtrate was stored in a 100 mL Schott bottle. The filter cake and 100 mL 0.01 M HCl were then added to a 100 mL Schott flask and were incubated in a rotary shaker at 150 rpm and 40 °C for 2 hours.
After 2 hours, the filter cake was washed with 20 mL sterile distilled water through a No 1 Whatman filter paper using in a Büchner funnel under vacuum. The filter cake was then added to the inactive MBR and filled up to 100 mL using the effluent filtrate solution. The remaining filtrate solution was added to the intermediate bottle.

3.5. Analytical methods

3.5.1. Sample preparation

All the samples, except the samples which were used for the heavy metal uptake by biomaterials and pseudo-catalyst inhibition and deactivation studies, were centrifuged at 13 000 rpm for 5 minutes using a Haraeus Megafuge 1.0 and filtered through a 0.22 µm Cameo filter before analysis. For the heavy metal uptake by biomaterials and pseudo-catalyst inhibition and deactivation of C. sinensis pomace study samples and the biomaterial suspension in the deconstructed two-stage immersed MBRs were first filtered through a pre-weighed filtered No 1 Whatman filter paper using a Büchner funnel under vacuum and dried at 80 °C for 24 hours to ascertain the dry mass of the biomaterial.

3.5.2. General water characteristics

An Oakton PCSTestr 35 Waterproof Multiparameter Tester was used to measure the pH, temperature, TDS and conductivity, and calibrated using the respective standards every 24 hours.

3.5.3. Inductively Coupled Plasma Atomic Emission Spectroscopy

The Ni, Zn and Cu concentrations in the filtrate were analysed using a Thermo iCap 6300 Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). The instrument was calibrated using the National Institute of Standards and Technology (NIST) traceable calibration standards from Merck, and the accuracy of results were verified using a separate NIST traceable standard.

3.5.4. Fourier Transform Inferred spectrophotometry

The functional groups of samples were analysed using a Perkin Elmer Fourier Transform Inferred (FTIR) spectrum 1000 spectrophotometer from 4000-400 cm⁻¹. In order to do this, a KBr disk was prepared by filtering and pressing a uniform mixture of sample and KBr under 8000 tonnes of pressure for 20 minutes.
3.5.5. Visible UV spectrophotometry

Total reduced sugar (TRS), uronic acids, CHOO⁻ and citric acid were quantified according to methods developed by Miller (1959), Filisetti-Cozzi and Carpita (1991), Taylor and Buchanan-Smith (1992), Sleat and Mah (1984) and Marier and Boulet (1958), respectively, using a Jenway 6715 UV/visible spectrophotometer.

3.5.6. Photometric test kits

Merck cyanide (CN⁻) (09701) and Merck ammonium (NH₄⁺-N) (00683) test kits and colour (method code of 032) were used to quantify the [T-CN], [F-CN] and [WAD-CN], [NH₄⁺] and colour in Pt/Co using a NOVA 60 spectroquant.
CHAPTER 4
RESULTS AND DISCUSSION
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RESULTS AND DISCUSSION

4.1. Characterisation of electroplating effluent

4.1.1. Introduction

An electroplating facility located in the Western Cape, South Africa, was discharging its effluent through the municipal systems without any treatment or analysis to ensure that it was meeting municipal discharge standards. The electroplating facility has a continuous supply of potable water (≈ 200 L/hour) to the washing baths and the effluent is then discharged at the same rate into a municipal drain. The electroplating of workpieces is for both decorative and protective purposes and utilises Ni, Zn and/or Cu plating metals. Heavy metal CN complexes are used as salts in the electrolyte for the electrolytic process. It is, thus, prudent to determine the degree of toxicity of the discharged effluent to determine if it is safe for municipal discharge and to monitor fluctuations in the contaminants over a period of time. The disposal of heavy metals and CN contaminants in effluent poses environmental concerns as a result of the bio-accumulative properties of the contaminants (Sud et al., 2007). Therefore, the contaminate characterisation of the electroplating effluent is paramount as the nature of the contaminants in the effluent would necessitate specific bioremediation methods.

4.1.2. Aims

The specific aims included the following:

- To determine the composition of the electroplating effluent.
- To compare the effluent with the municipal discharge standards.

4.1.3. Effluent analysis

The average composition of the electroplating effluent, based on 20 samples which were taken every 2 days over 40 days and the parameters of which would affect the operation if the effluent were reused directly, together with the respective mean and standard deviations are presented in Table 4.1. According to the standards presented in Table 4.1, the quality of the electroplating effluent did not meet the standards required for discharge into the municipal system.
### Table 4.1: Comparison between electroplating effluent composition with municipal discharge and potable water standards

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Municipal discharge standards (South Africa, 2006)</th>
<th>Potable water standards (SANS 241)</th>
<th>Electroplating effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>≥ 0 °C and ≤ 40 °C</td>
<td>Not indicated</td>
<td>22.50 (± 12.50) °C</td>
</tr>
<tr>
<td>pH value (25 °C)</td>
<td>≥ 5.5 and ≤ 12</td>
<td>≥ 5 and ≤ 9.7</td>
<td>10.46 (± 0.88)</td>
</tr>
<tr>
<td>Colour</td>
<td>Not indicated</td>
<td>≤ 12 Pt-Co</td>
<td>8.61 (± 1.15) Pt-Co</td>
</tr>
<tr>
<td>Electrical conductivity (25 °C)</td>
<td>≤ 500 mS/m</td>
<td>≤ 170 mS/m</td>
<td>145.73 (± 9.48) mS/m</td>
</tr>
<tr>
<td><strong>Chemical substances</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total dissolved solids (TDS)</td>
<td>≤ 4000 mg/L</td>
<td>≤ 1200 mg/L</td>
<td>998.83 (± 63.85) mg/L</td>
</tr>
<tr>
<td>Total cyanides as CN</td>
<td>≤ 20 mg/L</td>
<td>≤ 0.07 mg/L</td>
<td>149.11 (± 50.75)</td>
</tr>
<tr>
<td>Total sugars as glucose</td>
<td>≤ 1500 mg/L</td>
<td>Not indicated</td>
<td>Not detected</td>
</tr>
<tr>
<td>Total ammonia as N</td>
<td>Not indicated</td>
<td>≤ 1.5 mg/L</td>
<td>Not detected</td>
</tr>
<tr>
<td><strong>Heavy metals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copper as Cu</td>
<td>≤ 20 mg/L</td>
<td>≤ 2 mg/L</td>
<td>45.19 (± 25.89) mg/L</td>
</tr>
<tr>
<td>Zinc as Zn</td>
<td>≤ 30 mg/L</td>
<td>≤ 5 mg/L</td>
<td>9.05 (± 5.26) mg/L</td>
</tr>
<tr>
<td>Nickel as Ni</td>
<td>≤ 5 mg/L</td>
<td>≤ 0.07 mg/L</td>
<td>8.12 (± 4.78) mg/L</td>
</tr>
</tbody>
</table>

The main issues of concern were the T-CN and heavy metal (Ni, Zn and Cu) concentrations which also showed significant fluctuations as a function of time. This was directly related to the type of heavy metal coating(s) that was being carried out during the period of sampling. Upon further analysis, 96.5% of the T-CN being discharged was F-CN, 149.11 (± 9.31) mg F-CN/L and 5.25 (± 0.64) mg WAD-CN/L. This, in turn, meant that this parameter is of the utmost importance as a result of its extreme toxicity, including its volatility, followed by the heavy metals (Ni, Zn and Cu).

### 4.1.4. Summary

The analysis of effluent from the electroplating facility has highlighted the importance of developing bioremediation methods for the CN and heavy metal contaminants which were prevalent in this particular effluent. However, the substantial concentration of these contaminants would require a robust system of design while the level and fluctuations of the contaminant concentrations could pose a significant challenge, especially for an active microbial bioremediation stage. It was, thus, essential to apply a preliminary inactive bioremediation stage prior to an active microbial bioremediation stage as this preliminary stage should limit the impact of the fluctuations in the contaminant concentrations. In addition, the isolation of a microorganism that was tolerant to the level of contaminant present in the effluent was required. Although, it was important that the system be designed in such a way that it not only converts CNs, but also allows the removal and recovery of the heavy metals.
4.2. Identification and determination of the free cyanide tolerance of isolate

4.2.1. Introduction

In view of the ability of *Aspergillus* spp. to hydrolyse and metabolise agricultural products and CN, it was deemed necessary to isolate a strain of *Aspergillus* sp. from the electroplating environment. This was primarily as a result of the fact that the isolate had displayed a unique CN tolerance because of its being isolated from a CN containing environment.

An *Aspergillus* sp., which displayed the characteristic black conidiophores of the *Aspergillus section Nigri*, was isolated from the electroplating facilities’ effluent discharge. The identification of the strain was cumbersome because of the similarity between the species’ morphological and molecular characteristics to *Aspergillus niger* (*A. niger*). It was, thus, essential to determine an accurate method to identify the isolate. Similarly, in view of the high toxicity and the concentrations of F-CN in the electroplating effluent, the assessment of F-CN tolerance for the isolate was of paramount importance.

4.2.2. Aims

The specific aims included the following:

- To identify the *Aspergillus* sp. isolate using morphological and molecular methods
- To determine the F-CN tolerance of *Aspergillus* sp. isolate.

4.2.3. Free cyanide tolerance of isolate

The tolerance of F-CN by the isolate was initially assessed using a F-CN concentration up to 500 mg F-CN/L (see Figure 4.1). There was a clear decline in the growth of the fungus as the F-CN concentration was increased. However, appreciable growth occurred when the strain was grown in F-CN PDA plates in which concentrations of up to 200 mg F-CN/L were used, with rapidly declining growth when the F-CN concentration exceeded 300 mg F-CN/L. Minimal growth was observed up to 430 mg F-CN/L, an indication of the fungus’s maximum F-CN tolerance. The implication of this finding was that, if effluent contains F-CN concentrations exceeding a range of 400 to 500 mg F-CN/L, this may result in the reduction of the functionality of the fungal metabolic processes and, thus, in its growth and its potential for F-CN bioremediation.
Figure 4.1: F-CN tolerance analysis of the isolate cultivated on F-CN PDA plates at 37 °C for 5 days
4.2.4. Identification of the isolate

In order to observe the morphological characteristics of the fungus, the isolate was inoculated onto MEA and CYA plates. Based on its growth rate, the fungus was presumptively identified as *A. awamori*. *A. awamori* was reported to show rapid growth on the CYA as compared to *A. niger* which exhibited restricted growth (Santos *et al*. , 2013; Varga *et al*., 2011). However, the growth and sporulation on MEA was better than on CYA in the case of both *A. niger* and *A. awamori*.

![Figure 4.2: Top and bottom views of the isolate cultivated on (i & ii) MEA and (iii & iv) CYA plates at 26 °C for 7 days.](image)

The fruiting bodies were mounted in lactic acid before they were observed microscopically under oil immersion (Varga *et al*., 2011). The conidial heads for *A. awamori* were not well-defined columns as compared with the conidia heads observed for *A. niger*. The strain showed colony characteristics of both *A. niger* and *A. awamori*. On a general note, the conidiophores and conidia of *A. awamori* and *A. niger* are similar and morphologically indistinguishable (see Figure 4.2) (Varga *et al*., 2011).
Figure 4.3: (i & ii) Typical *Aspergillus* conidiophores with a radial head and (iii & iv) roughened, round conidia with regular low ridges and bars observed microscopically under oil immersion.

The molecular identifications were performed to confirm the identification of the isolate, denoted as *Aspergillus* CPUT, by initially using PCR analysis of the ITS, β-tubulin and calmodulin gene regions individually (see Figure 4.4). However, this analysis did not result in a clear indication that would enable the researcher to determine the species of the isolate with any confidence.

According to Varga *et al.* (2011), the only way in which to separate these species was through a combined gene phylogenetic analysis of ITS, β-tubulin and calmodulin gene regions (see Figure 4.5). The combined gene region analysis indicated that the *Aspergillus* strain was similar and, indeed, identical to the sequence of *A. awamori* strains.
Figure 4.4: NJ tree based on the analysis of the (i) ITS, (ii) β-tublin and (iii) calmodulin gene regions
Figure 4.5: NJ tree based on the analysis of combined ITS–β-tublin–calmodulin gene regions
4.2.5. Summary

It was shown that the isolate had a significant tolerance to F-CN of 430 mg F-CN/L, although the significant decline in microbial growth after 200 mg F-CN/L showed the critical tolerance point of the F-CN for the microorganism. Accordingly, for application purposes, it was deemed advisable that the isolate be applied to a system with F-CN concentration of ≤ 200 mg F-CN/L for the bioremediation of the electroplating effluent as the F-CN concentration in the effluent was below the critical inhibiting F-CN concentration. Challenges encountered in the identification of Aspergillus sp. included the fact that morphological studies are unable to provide a sound method for identifying strains in the Aspergillus genus. However, the isolate displayed the colony characteristic in which A. niger and A. awamori share, although an anomaly in the differing morphology of the conidia heads which A. niger and A. awamori also share was not observed. This may be as a result of a morphological mutation in the isolate and molecular studies would be required to differentiate these strains accurately. However, a combined ITS, β-tubulin and calmodulin gene region analysis was required to identify the isolate accurately as A. awamori.

4.3. Viability of agricultural residues

4.3.1. Introduction

The significant quantities of agricultural residues that are produced annually have triggered research designed to overcome the challenges associated with the treatment or utilisation of such waste materials. The advantages of using agricultural residues are that they are cheap, abundant and also environmentally and economically beneficial. They may be used in the production of value-added products and as bioremediation materials for toxins. This, in turn, reduces the direct waste disposal which impacts negatively on the environment (Nigam & Pandey, 2009; Mitchell et al., 2006). Agricultural residues such as C. sinensis pomace, M. domestica pomace, Z. mays cob and S. tuberosum peel are major contributors to the agricultural residues which are produced annually. These agricultural residues are produced in large quantities and may be used both as a feedstock for microbial operations as a result of their high carbohydrate content and also as biomaterials for the bioremediation of contaminants. There have, however, been limited studies conducted into the utilisation of these agricultural residues for a coupled supplementation system to support microbial growth and for the bioremediation of CN and heavy metals, particularly with regards to electroplating effluent. It is, thus, important to determine which agricultural residue would be best suited as a supplement for the A. awamori isolate and bioremediation of CN and heavy metals.
4.3.2. Aims

The specific aims included the following:

- To determine which agricultural residue is best suited to supplement *A. awamori* for the bioremediation of T-CN and heavy metals (Ni, Zn and Cu).
- To determine which agricultural residue is best suited for the bioremediation of T-CN and heavy metals (Ni, Zn and Cu).
- To compare the effect of hydrolysis on agricultural residues and the activity of the produced *A. awamori* biomass on the bioremediation of T-CN and heavy metals (Ni, Zn and Cu).
- To determine the changes in functional groups due to the activity of *A. awamori* biomass and hydrolysis on agricultural residues.

4.3.3. Free cyanide and heavy metal bioremediation

The performance of each of the agricultural residue (see Table 4.2) was based on three criteria in terms of how efficiently the: (1) T-CN was converted; (2) T-CN conversion by-products and sugar metabolism efficiencies; and (3) heavy metal (Ni, Zn and Cu) uptake by the biomaterials, respectively. The high cellulosic based agricultural residues, namely, *C. sinensis* pomace and *M. domestica* pomace, were the most efficient as compared to the high starch based agricultural residues, namely, *Z. mays* cob and *S. tuberosum* peel, for the three criteria used, irrespective of the degree of the hydrolysis of agricultural residue and the activity of the *A. awamori* biomass.

The conversion of T-CN was achieved only using the unhydrolysed and hydrolysed agricultural residues, as a result of their possession of free hydroxyl groups, and also active *A. awamori* biomass, as a result of the enzymes it produces. The high cellulosic based agricultural residues were found to be 67.23% more effective for the conversion of T-CN than the high starch based agricultural residues due to those residues containing higher quantities of free hydroxyl groups (Li et al., 2008). However, the hydrolysis of the agricultural residue was shown to significantly increase the T-CN conversion by 74.95%, 74.40%, 64.30% and 63.38% for *C. sinensis* pomace, *M. domestica* pomace, *Z. mays* cob and *S. tuberosum* peel, respectively. Nevertheless, the application of agricultural residues for T-CN conversion indicated that a secondary bioremediation stage was required for the removal of the by-products from the conversion of T-CN, for example an active microbial bioremediation stage.
Table 4.2: Effect of hydrolysis of agricultural residue and hydrolysed agricultural residue supplementation for *A. awamori* for T-CN conversion, T-CN conversion by-products and sugar metabolism efficiencies and heavy metal (Ni, Zn and Cu) uptake

<table>
<thead>
<tr>
<th>Agricultural residue</th>
<th>Comparative factors</th>
<th>T-CN conversion (%)</th>
<th>N-NH₄⁺ metabolism (%)</th>
<th>CHOO⁻ metabolism (%)</th>
<th>TRS metabolism (%)</th>
<th>Ni uptake (mg/g)</th>
<th>Zn uptake (mg/g)</th>
<th>Cu uptake (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrus sinensis pomace</td>
<td>Degree of hydrolysis</td>
<td>Unhydrolysed</td>
<td>26.77</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>0.30</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>Hydrolysed</td>
<td>35.62</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>0.61</td>
<td>0.56</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>Nutrient supplement</td>
<td>Active</td>
<td>32.74</td>
<td>65.12</td>
<td>100.00</td>
<td>4.88</td>
<td>5.87</td>
<td>4.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inactive</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>10.63</td>
<td>9.55</td>
<td>10.05</td>
</tr>
<tr>
<td>Malus domestica pomace</td>
<td>Degree of hydrolysis</td>
<td>Unhydrolysed</td>
<td>23.45</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>0.26</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Hydrolysed</td>
<td>30.53</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>0.43</td>
<td>0.40</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>Nutrient supplement</td>
<td>Active</td>
<td>27.97</td>
<td>67.26</td>
<td>100.00</td>
<td>4.73</td>
<td>5.62</td>
<td>4.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inactive</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>10.10</td>
<td>8.35</td>
<td>8.55</td>
</tr>
<tr>
<td>Zea mays cob</td>
<td>Degree of hydrolysis</td>
<td>Unhydrolysed</td>
<td>5.53</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>0.26</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Hydrolysed</td>
<td>15.49</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>0.30</td>
<td>0.27</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>Nutrient supplement</td>
<td>Active</td>
<td>25.87</td>
<td>69.81</td>
<td>100.00</td>
<td>2.70</td>
<td>3.93</td>
<td>4.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inactive</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>9.79</td>
<td>9.85</td>
<td>10.47</td>
</tr>
<tr>
<td>Solanum tuberosum peel</td>
<td>Degree of hydrolysis</td>
<td>Unhydrolysed</td>
<td>4.86</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>0.25</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>Hydrolysed</td>
<td>13.27</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>0.25</td>
<td>0.23</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Nutrient supplement</td>
<td>Active</td>
<td>22.08</td>
<td>72.41</td>
<td>100.00</td>
<td>2.53</td>
<td>3.73</td>
<td>2.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inactive</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>9.28</td>
<td>9.51</td>
<td>8.58</td>
</tr>
</tbody>
</table>
The T-CN conversion by active A. awamori biomass was, on average, 20.01% higher using the high cellulosic based agricultural residue extracts as compared to the high starch based agricultural residue extracts. High cellulosic agricultural residues are an effective feedstock for microbial operations because of their high cellulose and hemicellulose, which yield a variety of sugars when hydrolysed and also as a result of their low lignin composition as compared to other agricultural residues (Nigam & Pandey, 2009; Sud et al., 2008; Mitchell, et al., 2006). This was evident in the sugar metabolism by the fungus, which indicated a preference for carbohydrates derived from high cellulosic rather than high starch substrates. However, despite significant T-CN conversion by the fungus, the minimal sugar metabolism observed indicated that minimal supplementation was required for the fungus and this may, in turn, be advantageous for large scale processes. Nevertheless, the metabolism of the \( \text{NH}_4^+ \) was higher when the A. awamori biomass was supplemented with the high starch based agricultural residue extracts rather than the high cellulosic based agricultural residue with CHOO\(^-\) being readily metabolised. The readily metabolisable nature of the CHOO\(^-\) may also have contributed to the minimal sugar metabolism from the agricultural supplements which was observed. The larger quantities of T-CN, which was converted, resulted in larger quantities of \( \text{NH}_4^+ \) being in solution, and, thus, needing to be metabolised. Similarly, the fungus may also have had a preference for T-CN conversion rather than \( \text{NH}_4^+ \) metabolism.

The uptake of heavy metals was achieved using all biomaterials, with varying degrees of success as a result of surface morphology and the functional groups associated with the biomaterials. The average heavy metal uptake was 95.72% (Ni), 95.98% (Zn) and 95.72% (Cu) more efficient using the A. awamori biomass rather than the agricultural residue, irrespective of the degree of hydrolysis of the agricultural residue. The filamentous nature of the fungi resulted in a large surface area for heavy metal binding and, thus, enhanced heavy metal uptake. The hydrolysis of the agricultural residue was shown to significantly increase the heavy metal uptake by 49.27% (Ni), 51.14% (Zn) and 50.93% (Cu), 37.87% (Ni), 39.31% (Zn) and 38.70% (Cu), 12.18% (Ni), 14.15% (Zn) and 13.07% (Cu) and 9.38% (Ni), 11.22% (Zn) and 10.11% (Cu) for C. sinensis pomace, M. domestica pomace, Z. mays cob and S. tuberosum peel, respectively. The overall acid hydrolysis of the agricultural residue resulted in the modification of the functional group components in the residue structure, thus increasing the porosity in the agricultural residue’s structure (see Figure 4.6). This, in turn, increased the accessibility of the remaining functional groups, thus improving heavy metal binding, a phenomenon also observed by Li et al. (2008).
The inactive A. *awamori* biomass had a higher heavy metal uptake as compared to the active A. *awamori* biomass of 18.25% (Ni), 20.85% (Zn) and 19.13% (Cu), 18.14% (Ni), 20.83% (Zn) and 18.92% (Cu), 14.49% (Ni), 16.26% (Zn) and 15.14% (Cu) and 14.36% (Ni), 16.19% (Zn) and 15.08% (Cu) using *C. sinensis* pomace, *M. domestica* pomace, *Z. mays* cob and *S. tuberosum* peel extract supplementation, respectively.

Despite the fact that heavy metals comprise a vital aspect of cellular functions, they are required in trace amounts only. Heavy metal concentrations which exceed the concentrations required for cellular metabolism negatively affect active biomass and this may result in biomass lysis. This is one of the advantages associated with using inactive biomass as the heavy metal uptake is based solely on the availability of the heavy metal binding components in the inactive biomass.

### 4.3.4. Functional groups analyses

The effect of hydrolysing the agricultural residues, the activity of the A. *awamori* biomass, the type of growth media used for the biomass cultivation, as well as extremely alkaline (pH 12) and elevated temperature (50 °C) conditions, may alter the biomass chemical characteristics, thereby affecting its performance. This study found insignificant changes in the functional groups in the biomaterials, irrespective of the biomaterial and hydrolysis/activity, even when the biomaterials had been exposed to alkaline conditions (pH of 12) and elevated temperature (50 °C). However, several studies have indicated that the chemical modification of functional groups in biomaterials may be achieved under higher temperatures (≥ 80 °C) or when coupled with chemical treatment (Li *et al*., 2008; Kapoor & Viraraghavan, 1998). Similarly, the thermal treatment used to render the A. *awamori* biomass inactive did not cause any detectable breakdown or conversion of the functional groups.

As shown in Figures 4.6 and 4.7, the FTIR spectra depict a number of spectra peaks which indicate the types of functional groups present in the biomaterials. The spectra peaks appearing at wavelengths of 3700–3600, 3200–3600, 3000–2850, 1745, 1320–1210, 1250–950 and 540–500 cm⁻¹ are a characteristic of the O-H groups associated with free hydroxyl and phenol groups, O-H stretch in the H-bonded hydroxyl of alcohol and phenol groups, C-H stretch of the alkane group, C=O stretch of the 5-membered ketone group, C-O stretch of the acetyl group, P-H bends of the phosphine group and S-S disulphide group, respectively.
Figure 4.6: FTIR spectra of various unhydrolysed and hydrolysed agricultural residues

- **Unhydrolysed C. sinensis pomace**
- **Hydrolysed C. sinensis pomace**
- **Unhydrolysed M. domestica pomace**
- **Hydrolysed M. domestica pomace**
- **Unhydrolysed Z. mays cob**
- **Hydrolysed Z. mays cob**
- **Unhydrolysed S. tuberosum peel**
- **Hydrolysed S. tuberosum peel**

**Wavelength (cm⁻¹)**

Figure 4.7: FTIR spectra of *A. awamori* biomass cultivated using various agricultural residue extracts

- **C. sinensis pomace extract**
- **M. domestica pomace extract**
- **Z. mays cob extract**
- **S. tuberosum peel extract**

**Wavelength (cm⁻¹)**
There were changes observed in the amplitude/detection of the functional groups in the agricultural residue as a result of hydrolysis and the *A. awamori* biomass grown using various agricultural residue extracts. The type of growth media may have a significant impact on the *A. awamori* biomass functionality, particularly when the high cellulosic based agricultural residue extracts was used as compared with the high starch based agricultural residue extracts. A reduction in the amplitude of the functional groups were detected in the all the agricultural residues after hydrolysis as a result of the partial liberation of components into solution, only the high cellulosic agricultural residues showed undetectable 5-membered ketone and acetyl functional groups after acid hydrolysis. However, despite the reduction in the functional groups in the agricultural residues, the increased surface area, as a result of the hydrolysis of the agricultural residues, was shown to be the major contributor to increased heavy metal uptake capacity as compared to the unhydrolysed agricultural residues. It was possible to detect a 5-membered ketone and acetyl functional groups in the *A. awamori* biomass which was cultivated using the high cellulosic agricultural residue extract as compared to the high starch agricultural residue extract. The detection of these groups in the biomass and their reduction from their respective agricultural residues after hydrolysis was an indication that these highly reactive functional groups had been incorporated into the biomass, thus increasing the potential heavy metal uptake of the biomass.

### 4.3.5. Summary

The utilisation of high cellulosic based agricultural residues, namely, *C. sinensis* pomace and *M. domestica* pomace, was shown to be the best type of agricultural residue for T-CN conversion and heavy metals (Ni, Zn and Cu) uptake, supplement for T-CN conversion and the subsequent by-products metabolism and biomass development when compared to the high starch based agricultural residues, namely, *Z. mays* cob and *S. tuberosum* peel. The order of preference regarding which agricultural residue to use was as follows: *C. sinensis* pomace > *M. domestica* pomace > *Z. mays* cob > *S. tuberosum* peel. Nevertheless, the efficiencies for the respective high cellulosic and starch based agricultural residues were extremely similar and, thus, *M. domestica* pomace could be used as a substitute for *C. sinensis* pomace if the latter were unavailable. The use of high cellulosic based agricultural residues also presented with unique functional groups, namely, 5-membered ketone and acetyl functional groups, which were incorporated into the *A. awamori* biomass which was liberated from the acid hydrolysis of the agricultural residue. One of the important factors that require further analysis is the effect of pH and temperature on both the CN conversion and heavy metal uptake efficiency of the *C. sinensis* pomace and *A. awamori* biomass to aid in the development of an integrated system for the bioremediation of CNs and heavy metals.
4.4. Sensitivity analysis for free cyanide and heavy metals bioremediation

4.4.1. Introduction

One of the biggest contributors of agricultural residues results from citrus fruit processing. Citrus fruit processing has an approximate process quantity of 31.2 million tonnes every year globally and generates approximately 15.6 million tonnes of citrus residue, of which 75% (w/w) is *C. sinensis* pomace, thus making it the largest contributor of agricultural residues (Jarrell, 2012; Li *et al*., 2008). The hydrolysis of *C. sinensis* pomace may yield significant quantities of neutral sugars, such as glucose and fructose, with lower yields of arabinose, galactose, xylose and trace quantities of other neutral sugars, as well as uronic acids, with galacturonic acid being the predominate uronic acid liberated with trace quantities of other uronic acids (Grohmann *et al*., 1995). The characteristic quality of *Aspergillus* spp., such as *A. awamori*, to hydrolyse and metabolise carbohydrates in agricultural produce and CN is extremely useful and, thus, the availability of these sugars may sustain the growth of *A. awamori* (Gupta *et al*., 2010; Papagianni, 2007; Mitchell *et al*., 2006).

However, the development of an environmentally friendly bioremediation process to significantly bioremediate CNs and heavy metals requires a critical analysis of those factors that influence the respective bioremediation efficiencies, particularly pH and temperature (Igwe *et al*., 2011; Gupta *et al*., 2010; Eccles, 2000). It was, thus, important to determine how sensitive the bioremediation mechanism was to pH and temperature as most industrial treatment operations have varying conditions upon discharge. There is also a need for the development of an integrated bioremediation process. Limited studies have been performed to assess the bioremediation of CNs and heavy metals under alkaline pH conditions, which typify most industrial effluent containing these contaminants, for example, the effluent from the electroplating industry.

4.4.2. Aims

The specific aims included the following:

- To determine the effect of pH and temperature on the bioremediation of F-CN and heavy metals (Ni, Zn and Cu) using *C. sinensis* pomace and *A. awamori* biomass.
- To determine the effect of the hydrolysis of *C. sinensis* pomace and the activity of *A. awamori* biomass on the bioremediation of F-CN and heavy metals (Ni, Zn and Cu).
- To compare the bioremediation efficiencies and scrutinise the utilisation of optimum conditions for a continuous bioremediation process.
4.4.3. Free cyanide bioremediation

As shown in Figure 4.6, the free hydroxyl functional groups present in *C. sinensis* pomace, provided a pseudo-catalytic conversion for CNs. The rate of pseudo-catalysis of F-CN by both unhydrolysed and hydrolysed *C. sinensis* pomace exhibited a linear increase with respect to temperature and pH (see Figure 4.8). Although it is known that temperature is one of the most common and critical factors influencing the rate of reaction, it was observed that some of the pseudo-catalytic kinetics were significantly influenced by the pH of the solution. The maximum F-CN conversion of 17.82% and 62.48% using unhydrolysed and hydrolysed *C. sinensis* pomace was achieved at pH 12 and a temperature of 50 °C, respectively. The deprotonation of the free hydroxyl functional group is a combined effect of the alkaline pH, which weakens the O-H bond, and the presence of a strong base, such as F-CN. Similarly, increases in pH result in higher concentration of the hydroxide ions which form part of the F-CN reaction mechanism, thus increasing the conversion. Similarly, the increase in temperature results in an increased rate of reaction as the substrate, in this case, F-CN, has more free energy, thus causing increased movement and collisions involving the agricultural residue, in this case, the *C. sinensis* pomace, which facilitates the reaction.

![Figure 4.8: F-CN conversion for (i) unhydrolysed and (ii) hydrolysed *C. sinensis* pomace](image)

<table>
<thead>
<tr>
<th>Table 4.3: Regression parameters (b&lt;sub&gt;i&lt;/sub&gt;), goodness of model fit (R&lt;sup&gt;2&lt;/sup&gt;) and probability (ϕ) values for coded variables (X&lt;sub&gt;i&lt;/sub&gt;) responses for F-CN conversion using unhydrolysed and hydrolysed <em>C. sinensis</em> pomace</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-CN conversion</td>
</tr>
<tr>
<td>b&lt;sub&gt;0&lt;/sub&gt; ± ε</td>
</tr>
<tr>
<td>b&lt;sub&gt;1(1)&lt;/sub&gt;</td>
</tr>
<tr>
<td>b&lt;sub&gt;1(2)&lt;/sub&gt;</td>
</tr>
<tr>
<td>R&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>ϕ</td>
</tr>
</tbody>
</table>
Furthermore, hydrolysis of the C. sinensis pomace increased the pseudo-catalytic conversion of F-CN by 3.86 fold as compared to the unhydrolysed C. sinensis pomace. The hydrolysis of the C. sinensis pomace results in the release of components in the agricultural residue, thus increasing the surface area of the agricultural residue by making it more porous, a process which facilitated the availability of the hydroxyl functional groups which facilitate the protonation of the CN group. The conversion of F-CN by the A. awamori isolate was highly dependent on both pH and temperature and exhibited a characteristic parabolic conversion trend, thus indicating the sensitivity of the enzymes to pH and temperature. Optimal conditions were observed at pH 8.75 and 37.02 °C with these conditions achieving a F-CN conversion of 62.37%. However, pH and temperature conditions beyond this point inhibit the enzymes, thus reducing the activity of the enzyme. It was observed that ± 75% (w/w) of the NH₄⁺, undetectable CHO⁻ concentrations and ± 10% (w/w) of the TRS were metabolised by the fungus during the F-CN bioremediation.

![Graphs showing F-CN conversion, NH₄⁺ metabolism, and TRS metabolism efficiencies](image.png)

**Figure 4.9:** (i) F-CN conversion, (ii) NH₄⁺ metabolism and (iii) TRS metabolism efficiencies for A. awamori biomass

**Table 4.4:** Regression parameters (bₓ), goodness of model fit (R²) and probability (ϕ) values for coded variables (Xᵢ) responses for F-CN conversion and NH₄⁺ and TRS metabolism efficiencies using A. awamori biomass

<table>
<thead>
<tr>
<th>Efficiency</th>
<th>F-CN conversion</th>
<th>NH₄⁺ metabolism</th>
<th>TRS metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>b₀ ± ε</td>
<td>-359.50999</td>
<td>+202.39062</td>
<td>+40.00715</td>
</tr>
<tr>
<td>b₁(1)</td>
<td>+8.07503</td>
<td>-2.16771</td>
<td>+0.064540</td>
</tr>
<tr>
<td>b₁(2)</td>
<td>+62.31717</td>
<td>-19.95216</td>
<td>-7.32572</td>
</tr>
<tr>
<td>b₂(1)</td>
<td>-0.10668</td>
<td>+0.017014</td>
<td>+5.644x10⁻³</td>
</tr>
<tr>
<td>b₂(2)</td>
<td>-3.52097</td>
<td>+0.91273</td>
<td>+0.43794</td>
</tr>
<tr>
<td>b₃</td>
<td>-0.020236</td>
<td>+0.072994</td>
<td>-0.048583</td>
</tr>
<tr>
<td>R²</td>
<td>0.9828</td>
<td>0.8344</td>
<td>0.9835</td>
</tr>
<tr>
<td>ϕ</td>
<td>&lt;0.0001</td>
<td>0.0109</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
However, it was also determined that the *C. sinensis* pomace extract consisted of approximately 89.25% (w/w) of metabolisable sugars and 10.75% (w/w) of uronic acids, which cannot be metabolised by the fungus. Nevertheless, the metabolic efficiency of the TRS and NH$_4^+$ was favoured under conditions inversely proportional to those suitable for F-CN conversion, especially at higher temperatures (> 37.02 °C) and pH approaching the neutral range. It also suggested that the fungus may be gradually exposed to F-CN in order to acclimatis the metabolic processes so as to facilitate both the F-CN conversion and the rapid metabolism of metabolic by-products, particularly NH$_4^+$.

### 4.4.4. Heavy metal uptake

There was a similar heavy metal uptake affinity of the order Ni > Zn > Cu, irrespective of the type of the biomaterial used. The optimal uptake of Ni, Zn and Cu on active and inactive *A. awamori* biomass and unhydrolysed and hydrolysed *C. sinensis* pomace occurred at a pH of 12 and temperature of 37.91 and 39.78 °C, respectively.

**Figure 4.10**: Comparison of (i) Ni, (ii) Zn and (iii) Cu uptake onto unhydrolysed and hydrolysed *C. sinensis* pomace
Table 4.5: Regression parameters \((b_i)\), goodness of model fit \((R^2)\) and probability \((\phi)\) values for coded variables \((X_i)\) responses for Ni, Zn and Cu uptake using unhydrolysed and hydrolysed C. sinensis pomace

<table>
<thead>
<tr>
<th>Biomaterial</th>
<th>Unhydrolysed C. sinensis pomace</th>
<th>Hydrolysed C. sinensis pomace</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metal species</td>
<td>Ni</td>
<td>Zn</td>
</tr>
<tr>
<td>(b_0 \pm \varepsilon)</td>
<td>+49.92976</td>
<td>+46.55454</td>
</tr>
<tr>
<td>(b_1(1))</td>
<td>-0.049261</td>
<td>-0.04593</td>
</tr>
<tr>
<td>(b_1(2))</td>
<td>-11.74161</td>
<td>-10.94789</td>
</tr>
<tr>
<td>(b_2(1))</td>
<td>-1.626x10^{-3}</td>
<td>-1.516x10^{-3}</td>
</tr>
<tr>
<td>(b_2(2))</td>
<td>+0.64866</td>
<td>+0.60481</td>
</tr>
<tr>
<td>(b_3)</td>
<td>+0.021382</td>
<td>+0.019936</td>
</tr>
<tr>
<td>(R^2)</td>
<td>0.8734</td>
<td>0.8734</td>
</tr>
<tr>
<td>(\phi)</td>
<td>0.0109</td>
<td>0.0109</td>
</tr>
</tbody>
</table>

**Active A. awamori biomass**

(i) Ni uptake (mg/g) vs pH and Temperature (ºC)

(ii) Zn uptake (mg/g) vs pH and Temperature (ºC)

(iii) Cu uptake (mg/g) vs pH and Temperature (ºC)

**Inactive A. awamori biomass**

(i) Ni uptake (mg/g) vs pH and Temperature (ºC)

(ii) Zn uptake (mg/g) vs pH and Temperature (ºC)

(iii) Cu uptake (mg/g) vs pH and Temperature (ºC)

Figure 4.11: Comparison of (i) Ni, (ii) Zn and (iii) Cu uptake onto active and inactive A. awamori biomass
Table 4.6: Regression parameters ($b_i$), goodness of model fit ($R^2$) and probability ($\phi$) values for coded variables ($X_i$) responses for Ni, Zn and Cu uptake for active and inactive A. awamori biomass

<table>
<thead>
<tr>
<th>Biomaterial</th>
<th>Active A. awamori biomass</th>
<th>Inactive A. awamori biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metal species</td>
<td>Ni</td>
<td>Zn</td>
</tr>
<tr>
<td>$b_0 \pm \epsilon$</td>
<td>+63.0391</td>
<td>+57.54081</td>
</tr>
<tr>
<td>$b_{1(1)}$</td>
<td>+0.24349</td>
<td>-0.015517</td>
</tr>
<tr>
<td>$b_{2(1)}$</td>
<td>-4.272x10$^{-3}$</td>
<td>-1.410x10$^{-3}$</td>
</tr>
<tr>
<td>$b_{2(2)}$</td>
<td>+0.89932</td>
<td>+0.5378</td>
</tr>
<tr>
<td>$b_{3}$</td>
<td>+8.475X10$^{-3}$</td>
<td>+0.012247</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.8132</td>
<td>0.8272</td>
</tr>
<tr>
<td>$\phi$</td>
<td>0.0219</td>
<td>0.0187</td>
</tr>
</tbody>
</table>

Temperatures exceeding the optimum temperature resulted in poorer heavy metal uptake as a result of the attractive forces between the functional groups and heavy metals being weakened (Horsfall & Spiff, 2005). However, the heavy metal uptake of Ni, Zn and Cu on active and inactive A. awamori biomass was significantly higher than that reported in the literature for non-modified, unchemically treated active and inactive biomasses for Aspergillus spp. (Wang & Chen, 2009). The uptake of Ni, Zn and Cu on hydrolysed and unhydrolysed C. sinensis pomace was similar to that reported in the literature for C. sinensis peel (Li et al., 2008; Wan Hgah & Hanafiah, 2008).

The dominant functional group associated with C. sinensis pomace is the hydroxyl group which is, in turn, associated with an affinity for Ni, Zn and Cu species (Eccles, 2000). As observed in this study, for a multi-metal solution, the Cu uptake may be inhibited significantly by the presence of Ni and Zn (see Figures 4.10 and 4.11). The heavy metal uptake capacity was: inactive A. awamori biomass > active A. awamori biomass > hydrolysed C. sinensis pomace > unhydrolysed C. sinensis pomace. The hydrolysed C. sinensis pomace heavy metal uptake capacity was 52.61% (Cu), 47.07% (Ni) and 48.05% (Zn) more efficient than that observed for the unhydrolysed C. sinensis pomace (see Figure 4.10). Similarly, the inactive A. awamori biomass had a 21.29% (Ni), 21.22% (Zn) and 19.74% (Cu) higher heavy metal uptake capacity than the active A. awamori biomass (see Figure 4.11). Both the active and the inactive A. awamori biomass showed a significantly higher metal uptake capacity of 51.72% (Ni), 51.43% (Zn) and 70.93% (Cu) as compared to that of the unhydrolysed and hydrolysed C. sinensis pomace (see Figures 4.10 and 4.11).
4.4.5. Summary

The ability of \textit{C. sinensis} pomace and \textit{A. awamori} biomass to bioremediate CNs and heavy metals is of great potential in terms of its application in effluent treatment. However, despite the fact that active \textit{A. awamori} biomass possesses combined F-CN conversion, F-CN conversion by-products metabolism and heavy metal uptake functions, its sensitivity to fluctuations in the contaminants would affect the overall bioremediation efficiency. Similarly, when the concentration of contaminants exceeds the microorganism(s) tolerance level, the active bioremediation process which has been designed would be redundant. A primary inactive bioremediation stage to reduce fluctuation effects in contaminant concentrations is, therefore, of the utmost importance. In addition, it must be borne in mind that, although both \textit{C. sinensis} pomace and \textit{A. awamori} biomass displayed a common heavy metal uptake affinity (Ni > Zn > Cu), \textit{A. awamori} biomass had a significantly higher heavy metal (Ni, Zn and Cu) uptake capacity than \textit{C. sinensis} pomace. Nevertheless, hydrolysed and unhydrolysed \textit{C. sinensis} pomace would be a more suitable selection for a primary bioremediation stage as they have the dual capability to pseudo-catalytically convert F-CN and heavy metal (Ni, Zn and Cu) uptake. Hydrolysed \textit{C. sinensis} pomace showed the most potential in a primary inactive bioremediation stage as it exhibited significantly higher F-CN conversion and heavy metal (Ni, Zn and Cu) uptake capacity as compared to when it was in the unhydrolysed form while also producing an extract that could be used to supplement the active \textit{A. awamori} bioremediation stage. However, the application of the active \textit{A. awamori} biomass could be used primarily as an F-CN converter or metaboliser of the F-CN conversion by-products, particularly NH$_4^+$.

4.5. Two-stage membrane bioreactor design and continuous operation

4.5.1. Introduction

Despite the fact that the conventional methods are established technologies, they are not environmentally friendly and, thus, further development is required in order to reduce the high/costly reagent requirements (Gupta \textit{et al.}, 2010; Patil & Paknikar, 2000; Nesbitt, 1996). The recent increase in the popularity of the bioremediation of CN and heavy metals may be ascribed to the potential environmental friendly and sustainability that they offer. However, the use of active biomaterials is complicated by both their contaminant tolerance and also the fluctuations in their concentrations which affect the overall bioremediation efficiency. They also possess unique functions which are not present in inactive biomaterials.

It was, thus, deemed prudent to develop a MBR system that would be able to overcome these challenges and also validate the bioremediation process technology for industrial
effluent, such as electroplating effluent. A two-stage immersed MBR bioremediation system was designed to treat the electroplating effluent to an acceptable standard so that it could be reused or else it would be in compliance with the municipal discharge standards. The process utilised a primary inactive bioremediation stage using hydrolysed C. sinensis pomace for the conversion of CN and the removal of the heavy metal contaminants and also to reduce the impact of fluctuations. In addition, the process also utilised a secondary active bioremediation stage using active A. awamori biomass to convert and remediate any residual contaminants in the effluent. This was supplemented by a C. sinensis pomace extract, derived from the acid hydrolysis of the C. sinensis pomace. The efficiency of this particular integrated system for the bioremediation of CN and heavy metal bearing effluent at alkaline conditions has not been previously reported upon before.

4.5.2. Aims

The specific aims included the following:

- To determine the bioremediation efficiency of each process stage and also of the overall process.
- To determine whether it would be possible to reuse the treated effluent or whether it meets the municipal discharge standards.
- To recommend improvements to the two-stage MBR design and bioremediation efficiency.

4.5.3. Process design

The optimal temperature of 40 °C was selected as the global optimum temperature of the process as it was the most favourable for the CN conversion and heavy metal uptake process. In addition, the design of a continuous bioremediation system required that the CN containing effluent be gradually introduced into the secondary bioremediation stage in order both to facilitate the acclimatisation of the active A. awamori biomass and initiate the metabolic activities required in the process. The use of C. sinensis pomace extract had showed potential as a rich, carbon based supplementation source and the fungus would require minimal augmentation by this carbon source supplement, ≤ 0.1% (v/v), and this would be suitable on an industrial scale. Based on these results, it was possible to recommend a multi-step bioremediation process design in order to increase both the overall CN conversion and the rapid metabolism of the metabolic by-products, particularly NH4+. One of the key factors that could significantly affect the conversion of CNs is species that have a greater affinity for the free hydroxyl functional groups.
A common example is the presence of heavy metals, such as Ni, Zn and Cu, which have shown to have an affinity to attaching to the free sites on the hydroxyl functional groups (Wan Hgah & Hanafiah, 2008; Eccles, 2000). It was shown that it was possible to achieve the T-CN conversion of 62.48 % in metal-free 100 mg T-CN/L solutions at 40 °C and pH 12 after 48 hours of using the hydrolysed C. sinensis pomace. However, the conversion was reduced to 43.80% in solutions containing 10 mg/L of heavy metals (Ni, Zn and Cu) under the same conditions. This indicated a 26.35% reduction in the T-CN conversion as a result of the binding of the heavy metals to the hydroxyl functional groups, responsible for the deprotonation effect on the CN group. This phenomenon refers to a competing species resulting in the deactivation of the pseudo-catalyst. In view of the fact that it had been observed that negligible T-CN conversion or heavy metal uptake had been detected when the benzylated C. sinensis pomace was used, the pseudo-catalytic properties of the hydrolysed C. sinensis pomace in a primary bioremediation stage for the conversion CN and heavy metal uptake in effluents is feasible. In addition, this primary bioremediation stage also enables the subsequent generation of the hydrolysed C. sinensis pomace through the use of acid regeneration, such as 0.1 M HCl, to subsequently recover heavy metals and also protonate the hydrolysed C. sinensis pomace (Li et al., 2008; Horsfall & Spiff, 2005).

4.5.4. Continuous operation for the bioremediation of electroplating effluent

The two-stage MBR system ran for 12 days using the cyclical T-CN selected electroplating effluent feed. The average bioremediation efficiencies for the T-CN, F-CN, WAD-CN, Ni, Zn and Cu for the 12 days of continuous operation are as shown in Table 4.7.

**Table 4.7: Averaged bioremediation efficiency for CNs and heavy metals using the two-stage MBR process designed**

<table>
<thead>
<tr>
<th>Contaminate</th>
<th>Average bioremediation efficiency (%)</th>
<th>After first bioremediation stage</th>
<th>After second bioremediation stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-CN</td>
<td>76.37</td>
<td>99.55</td>
<td></td>
</tr>
<tr>
<td>F-CN</td>
<td>75.80</td>
<td>98.06</td>
<td></td>
</tr>
<tr>
<td>WAD-CN</td>
<td>65.16</td>
<td>90.67</td>
<td></td>
</tr>
<tr>
<td>Ni</td>
<td>93.26</td>
<td>99.92</td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>94.76</td>
<td>99.92</td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>95.37</td>
<td>99.91</td>
<td></td>
</tr>
</tbody>
</table>

The concentration of these respective contaminants for the feed, intermediate and product streams versus time are presented in Figure 4.12. In addition, an average of 10.64% and 7.39% higher conversion was achieved for F-CN than WAD-CN for the first and second bioremediation stages, respectively. This was as a result of the combined inhibiting effects of the heavy metals (Ni, Zn and Cu) on the conversion of WAD-CN as compared to F-CN.
There was an evident reduction of 85.48%, 45.11%, 47.86% and 49.93% in the fluctuations for T-CN, Ni, Zn and Cu concentrations after the primary bioremediation stage and this would have negatively affected the bioremediation efficiency of the secondary bioremediation stage. Similarly, the secondary bioremediation stage metabolised the CN converted by-products by 99.81% and 99.75% for CHOO$^-$ and NH$_4^+$, respectively, despite significant concentration fluctuations of these by-products in the intermediate stream (see Figure 4.13). However, the concentrations of these by-products in the product stream were at acceptable levels for municipal discharge.
The TRS concentration, comprising uronic acid and neutral sugars, in the intermediate stream was designed to supplement the secondary bioremediation stage, and exhibited a declining trend for the first 4 days of continuous operation, after which the trend reached a plateau (see Figure 4.14). Minimal residual quantities of TRS were transferred into solution from the hydrolysed *C. sinensis* pomace from the primary bioremediation stage into the secondary bioremediation stage.

However, the uronic acid concentration remained relatively constant throughout the continuous operation with the neutral sugars being the only metabolisable carbon source in the *C. sinensis* pomace extract. The metabolism of the neutral sugars and the biomass development exhibited a plateau after 6 days as the fungus was adapting to the continuous operation mode (see Figures 4.14 and 4.15). After 9 days of continuous operation, the TRS concentration in the product stream exhibited an asymptotic trend towards the uronic acid trend which was as a result of the near metabolism of the neutral sugars.
However, the trace quantities of TRS in the product stream do not pose any environmental danger while the TRS concentration meets the municipal discharge standards. Similarly, the low supplement requirements of the fungus using the *C. sinensis* pomace extract and the contaminant bioremediation observed showed potential for the process as an effective bioreactor system. The pH, conductivity, TDS and colour (see Figure 4.16) of the feed, intermediate and product streams were analysed for reuse purposes as these parameters all affect the reusability of the effluent.

![Figure 4.15: Dry biomass development versus time](image)

Figure 4.15: Dry biomass development versus time

![Figure 4.16: (i) pH, (ii) conductivity, (iii) TDS and (iv) colour of the feed, intermediate and product streams](image)

Figure 4.16: (i) pH, (ii) conductivity, (iii) TDS and (iv) colour of the feed, intermediate and product streams
The colour of the effluent was shown to be problematic as it significantly increased after the primary bioremediation stage as a result of the transfer of the pigments from the hydrolysed 
\textit{C. sinensis} pomace and \textit{C. sinensis} pomace extract to the effluent. The alkaline pH of the effluent also resulted in an increase in the colour of the effluent as a result of the chemical modification of these pigments which, in turn, resulted in a luminescent colour developing. However, there was a reduction in colour of the effluent as a result of the wash out by the incoming effluent into the system. The pH, conductivity and TDS are all interrelated and are directly proportional to each other and, thus, affected by the species present in the effluent. Thus, the reduction in the effluent contaminants resulted in a reduction in these parameters.

Nevertheless, the quality of the product stream, with regards to the T-CN and heavy metals (Ni, Zn and Cu) contaminants, does meet the municipal discharge standards. In addition, the generation of the hydrolysed \textit{C. sinensis} pomace was performed every 4 days, with an approximate loss of 0.25% of the hydrolysed \textit{C. sinensis} pomace as a result of the acid regeneration and the approximate recovery of Ni, Zn and Cu as shown in Table 4.8.

<table>
<thead>
<tr>
<th>Regeneration cycle</th>
<th>Ni</th>
<th>Zn</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99.13</td>
<td>99.12</td>
<td>99.04</td>
</tr>
<tr>
<td>2</td>
<td>98.94</td>
<td>98.92</td>
<td>98.41</td>
</tr>
<tr>
<td>3</td>
<td>98.46</td>
<td>98.44</td>
<td>97.91</td>
</tr>
</tbody>
</table>

The insignificant change in the recovery efficiency of the heavy metals (Ni, Zn and Cu) from the hydrolysed \textit{C. sinensis} pomace and the evident pseudo-catalytic conversion of the CNs after the regeneration of the hydrolysed \textit{C. sinensis} pomace highlighted the potential of its reusability and also the possibility of the coupled removal and conversion of heavy metals and CNs, respectively, on a large scale.

4.5.5. Summary

The two-stage MBR design proved both to be robust and also to reduce the fluctuations of CN and heavy metal contaminants using the primary bioremediation stage to facilitate the metabolism and removal of the contaminants in the electroplating effluent on a constant basis. However, the design succeeded only in treating the effluent for safe municipal discharge although the use of the post-treatment stage, such as reverse osmosis, to remove the remaining trace contaminants and colourants from the product stream will ensure that it meets the potable water standards and will be able to be reused.
This design has highlighted the potential of using agricultural residues as biomaterials and a nutrient source to supplement an active microbial bioreactor. This incorporates unique bioremediation abilities and metabolic characteristics not previously possible while it is also more cost effective than the conventional treatment methods. In addition, there was a relatively insignificant standard deviation (≤ 3.22%) detected in all the parameters measured in the continuous operation and this, in turn, indicates the on-going reproducibility in the bioremediation efficiency of this continuous process.
CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS
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5.1. Conclusions

The detection of significant concentrations of F-CN, Ni, Zn and Cu in the effluent generated from an electroplating facility is a matter of significant environmental concern. The characteristic alkaline conditions of this effluent typify most industrial effluents containing these contaminates. However, a limited number of studies have been performed to assess the coupled bioremediation of CNs and heavy metals, especially using biological methods. Selective isolation procedures were employed to isolate an Aspergillus sp., which displayed the characteristically black conidiophores of the Aspergillus section Nigri, from the electroplating facilities’ effluent discharge. The isolate’s F-CN tolerance showed that the critical concentration that indicated an appreciable inhibition in microbial growth was higher than that determined in the electroplating effluent. This characteristic was a fundamental requirement for the bioremediation of this particular effluent and its application in a bioremediation operation, although this had been expected as this isolate was isolated from an environment bearing CN contaminated effluent.

The identification of the isolate was conclusively determined as A. awamori through molecular analysis using combined gene region analysis. Despite the fact that the morphological analysis was not an accurate method to identify strains belonging to this particular genus, it was feasible to indicate an anomaly in the characteristic morphology of the conidial heads associated with this strain and which indicated a possible morphological mutation of the isolate.

Agricultural residues such as C. sinensis pomace, M. domestica pomace, Z. mays cob and S. tuberosum peel are generated in large quantities every year and may be utilised both as a nutrient supplement for the A. awamori isolate and as biomaterials for the bioremediation of CNs and heavy metals with varying degrees of success. Experimental analysis indicated conclusively that the high cellulosic based agricultural residues, namely, C. sinensis pomace and M. domestica pomace, were more suitable both for CN conversion and heavy metals uptake, including subsequent by-products metabolism and A. awamori biomass development as compared to the high starch based agricultural residues, namely, Z. mays cob and S. tuberosum peel.
In addition, the hydrolysis of the agricultural residue improved the pseudo-catalytic conversion and uptake of CN and heavy metals, respectively. Furthermore, the inactive *A. awamori* biomass had an improved heavy metal uptake, irrespective of the agricultural residue or extract utilised. Overall, the *C. sinensis* pomace proved to be the most suitable and was, therefore, the agricultural residue which was selected for further experimentation.

Upon further experimentation, it was found that the conversion of CN using *C. sinensis* pomace increased linearly with an increase in pH and temperature. In addition, the heavy metal uptake, CN conversion, CN conversion by-products and sugar (from the *C. sinensis* extract) metabolism by *C. sinensis* pomace and/or *A. awamori* biomass all indicated specific optimum parameters. However, although the use of a *C. sinensis* extract proved to be a rich and compatible source for the cultivation of the isolate for concurrent CN conversion, CN conversion by-products metabolism and heavy metal uptake, there was minimal metabolism of the sugars present in the *C. sinensis* extract – an indication of the minimal supplementation requirements of this particular isolate.

The two-stage immersed MBR bioremediation system successfully treated the electroplating effluent to meet municipal discharge standards. The primary bioremediation stage utilised hydrolysed *C. sinensis* pomace to bioremediate a majority of the CNs and heavy metals and to reduce the impact of fluctuations while a secondary bioremediation stage utilised active *A. awamori* biomass supplemented by a *C. sinensis* pomace extract to bioremediate the residual contaminants in the effluent.

The selection of a global operating temperature for the design with minimal external modification of the pH of the effluent resulted in the successful bioremediation of the contaminants in the effluent. In addition, it was found that the removal of heavy metals, which had inhibited the pseudo-catalytic conversion of the CN during the primary bioremediation stage, could be successfully controlled by the acid regeneration which removed and recovered heavy metals in order to reactivate the pseudo-catalytic potential of the hydrolysed *C. sinensis* pomace.

This design highlighted the potential of using an environmentally friendly and sustainable process for the bioremediation of CN and heavy metal contaminated effluent. Nevertheless, further research and modifications are required to improve the bioremediation efficiency of the design so that its product stream meets the potable water standards and can be reused.
5.2. Recommendations

The following recommendations are suggested for future research:

- Mathematically model the heavy metal biosorption/bioaccumulation, CN conversion, CN conversion by-products and sugar metabolism and growth kinetics in order to assist in forecasting MBR performance and design.
- Determine the optimisation of residence time, mixing rate and regeneration cycles to determine the optimum MBR operation.
- Determine the applicability of a polishing stage after the design proposed in order to remove trace contaminants.
- Determine the applicability and bioremediation efficiency of various industrial effluents that are characterised by CN and heavy metal contaminants.
- A scale-up design to determine the operative and economic feasibility of a large scale industrial bioremediation operation be explored.
REFERENCES


