BIODETOXIFICATION OF ORGANIC CONTAMINANTS USING MICROORGANISMS ISOLATED FROM GOLD (Au) MINE TAILINGS

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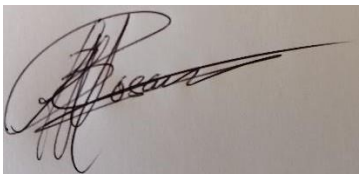
DECLARATION

I, Rashid Ahmed Mosam, declare that the contents of this dissertation represent my own unaided work, and that the dissertation has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the University of South Africa.



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ABSTRACT

Benzonitrile (C6H5C≡N) is a synthetic nitrile with vast applications as a solvent and intermediate in the manufacturing of drugs, perfumes, dyes, rubber, textiles, agrochemicals, resins, and specialty lacquers. Benzonitrile is the most used form of synthetic nitriles. The discharge of these synthetic nitriles directly into the environment via wastewater is an alarm for possible health hazard, pointing to the toxicity of these compounds some of which possess carcinogenic and mutagenic traits. Therefore benzonitrile (synthetic nitrile) waste requires treatment before being discharged into the environment. The physical and chemical treatment methods to treat nitrile contaminated waste is usually expensive and could lead to the production of toxic by-products that require further treatment. Hence bioremediation process which is an environmentally friendly, cost effective and does not produce toxic by-products is an effective alternative and preferred method of decontamination. The hypothesis of the study was that microorganisms isolated from Au mine tailings are able to degrade environmental contaminants (nitriles).

The study approach included isolating and identifying microorganisms from gold (Au) mine tailings. Followed by the extraction and identification of organic compounds foun in Au mine tailings. A solid-liquid extraction method was used with dichloromethane (DCM), ethyl acetate and hexane as extraction solvents. Compounds were identified via Liquid Chromatography quadrupole –Time-of-Flight Mass Spectrometry (LC-QTOF-MS), for non-volatile compounds, and Gas Chromatography Quadrupole Time of Flight Mass Spectrometry (GC-QTOF-MS), for volatile compounds. This study focused on the degradation of benzonitrile as the contaminant of interest. Preliminary biodegradation trials were conducts whereby the microorganisms were incubated in a minimal media (M.M) which contained aminobenzonitrile, as the sole source of carbon and nitrogen, for 72 hrs., shaking at 180 rpms, pH 7 and 30 °C. Ammonia was monitored as a product of nitrile degradation using Merck ammonium ($NH4^+$) (00683) test kit and quantified using a Merck Spectroquant Nova 60. A bacterial consortium was formed which consisted of bacterial species capable of degrading aminobenzonitrile thereby yielding ammonium. The bacterial consortium was subjected to optimization studies using response surface methodology (RSM) to achieve optimal physiochemical parameters. Biodegradation

products were identified using GC-MS and allowed for pathway determination based on products formed.

The isolated microorganisms from Au mine tailings belonged to the *Bacillus* and *Paenibacillus* genus as identified by the 16S rRNA gene sequencing. The microorganisms isolated from Au mine tailings were identified as *P. shunpengii, B. pumilus, B. safensis, B. thuringiesis* and *B. cereus*. A total of 27 organic compounds of interest were extracted and identified, compound classes with environmental significance such as; ethers, pyridines, pyrimidines, phenols and nitriles were amongst these compounds of interest. Preliminary biodegradation trials conducted indicated that all bacterial species isolated from Au mine tailings possess the ability to biodegrade aminobenzonitrile by detecting ammonium on the Merck spectroquant. The physical and chemical parameters of biodegradation of aminobenzonitrile were optimised using RSM with optimised conditions for temperature, pH and substrate (aminobenzonitrile) concentration being 35 °C, 9.46 and 50 mg/L respectively, with the maximum biological removal efficiency (BRE %) of 73% over a 72 hrs. period. 2-piperidinone was identified as a biodegradation product of aminobenzonitrile by the bacterial consortium, furthermore, 2-piperidinone is a cyclic amide which indicates that the bacterial consortium utilises the NHase enzymatic pathway of nitrile degradation.

Key words: Aminobenzonitrile; Biodegradation; Bioremediation; LC-QTOF-MS; GC-QTOF-MS; Response Surface Methodology; 2-piperidinone; Au mine tailings.

DEDICATION

This dissertation is dedicated to my late father, Feizal Mosam, whom I dearly miss. He instilled the importance of hard work and education which ultimately led me down this road. Although he passed on early during my masters studies, I still cherish and appreciate how he use to urge me to work hard and progress in order to achieve my goals in life. Thank you for believing in me.

Knowledge does not come but you have to go to it."

-Imam Malik

!

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LAYOUT OF DISSERTATION

The overall aim of the study was to isolate microorganisms (bacteria) from gold (Au) mine tailings and utilise these organisms for biodetoxification/bioremediation of organic contaminants such as benzonitriles.

The dissertation consists of the following chapters:

- **Chapter 1**, the introduction, contains background information on organic contaminants and bioremediation. The problem statement; hypothesis; aims and objectives are also stated in this chapter.
- **Chapter 2**, the literature review, provides information on gold mine tailings, microbes in a toxic environment and organic environmental contaminants. It also focuses on nitrile contamination and treatments; as well as benzonitrile contamination and treatment methods. The review provides information on the biological degradation of nitriles, focusing on the enzymes involved, their mechanisms of action and bacterial species that contain these enzymes. The chapter ends off with bioremediation technology providing background information; principles and advantages of bioremediation as a tool to combat contamination.
- **Chapter 3**, the research methodology, provides the materials and methods used to conduct the different experiments in achieving the objectives. This includes: the site description; isolation and identification of microbes found in Au mine tailings; extraction and identification of organic compounds in Au mine tailings; preliminary biodegradation trials and the optimisation of parameters for biodegradation.
- **Chapter 4**, the results and discussion, the performed experiments' results are discussed in this chapter.
- **Chapter 5**, the conclusion and recommendation, this section summarises the outcome and recommendations made for future studies.
- **References**, the references that were used to compile this study.
- **Appendices**

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LIST OF SYMBOLS

Nomenclature

Symbol	Description	Units
C_i	Initial concentration	mg/L
C_f	Final concentration	mg/L
m/z	Mass to charge ratio	-
RT	Retention time	Minutes (min)
R	Correlation coefficient	-
R^2	Determination coefficient	-
Y	Response variable	Units not defined
BRE	Biological removal efficiency	%

Subscripts

Symbol	Description	Units
A	Temperature	°C
B	pH	-
C	concentration	mg/L

GLOSSARY

Abbreviation	Description
ANOVA	Analysis of variance
Au	Gold
B. pumilus	*Bacillus pumilus*
B. safensis	*Bacillus safensis*
B. shunpengii	*Bacillus shunpengii*
B. thuringiesis	*Bacillus thuringiesis*
B. cereus	*Bacillus cereus*
CCD	Central composite design
DCM	Dichloromethane
DNA	Deoxyribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
ESI	Electron spray ionisation
EtOAc	Ethyl acetate
GC-QTOF-MS	Gas chromatography quadrupole-Time-of-Flight Mass spectrometry
He	Helium
hrs.	hours
LB	Luria Broth
LC-QTOF-MS	Liquid chromatography quadrupole-time-of-flight mass spectrometry
M.M.	Minimal media
NB	Nutrient broth
NH3	Ammonia
NH4	Ammonium
NHase	Nitrile hydratase
P. shunpengii	Paenibacillus shunpengii
RP-UHPLC	Reverse phase ultra high performance liquid chromatography
Rpm	Revolutions per minute
RSM	Response surface method

CHAPTER 1: INTRODUCTION

1.1 Background and Rationale

Hazardous chemicals are produced from industrial process waste products, which are directly discharged into sewers, rivers and wetlands. Industrial waste disposed in landfills or slag heaps may release substances that eventually seep into nearby rivers and dams. Mining activities produce waste and water pollutants in the form of acid mine drainage, which dissolves salts and mobilizes metals from mine workings and residual deposits. Acid mine drainage not only pollutes water sources, but it is also responsible for the negative environmental effects such as degradation of soil quality, aquatic habitat and introducing heavy metals into the environment (Musingafi and Tom, 2014). Fertilisers and pesticides from agricultural activities result in the pollution of water in addition to agrochemicals, salts and toxic leachates. Various agricultural activities are responsible for an increase in chemical oxygen demand (COD), ammonia-N, nitrogen, and phosphorus levels which are released into the water systems (Abbasi et al., 2014). Nitrogen and phosphates are widespread water pollutants. South Africa's wastewater treatment systems / plants are capable of treating nitrogen breakdown but lack any system for phosphates, therefore phosphates enter the water supply untreated. Phosphate pollutants pose a threat to the water system by altering the way in which a river system works, alter the functioning and working of a river system through eutrophication leading to algal blooms which may contain cyanobacteria that pose a risk to human and animal health (Colvin et al., 2016).

Natural water resources in South Africa are polluted by a variety of industries, including mining activities. Over the past 20 years, the water quality of South African rivers and dams has dramatically deteriorated due to pollution. Water quality throughout South Africa is affected in various ways which amongst others include; poorly treated sewage effluent from failing sewage treatment works, poor sanitation in informal settlements and rural areas, mining and ore processing activities particularly acid mine drainage, industrial effluents during product manufacturing, agricultural runoffs carrying fertilizer, pesticides and sediments (Colvin et al., 2016) and migration or distribution of mine tailings to surface waters.

Emissions from anthropogenic activities such as vehicle, industrial biomass burning emissions have resulted in the deterioration of air quality in South Africa and globally (Garland et al., 2017). Volatile organic compounds, such as benzene; toluene; ethylbenzene and xylene, are released to the environment via various anthropogenic activities as well (Lourens et al., 2011).

Nitriles are organic molecules containing a carbon double bonded to nitrogen (-C=N) functional group. Bacteria, fungi, plants, marine and terrestrial habitats are examples where nitriles are found naturally. Extensive use of nitriles in the industrial sector for the production of plastics, solvents and synthetic intermediates are examples of synthetically produced nitrile sources (Tanii, 2017). The pharmaceutical and herbicide industry also makes use of nitriles (Egelkamp et al., 2019; Fleming et al., 2010). Synthetic nitriles (Man-made) are widely used in the manufacturing of feedstock, extractants, solvents, drug intermediates, pharmaceuticals, and pesticides. They also form important intermediates in the organic synthesis of amines, amides, amidines, carboxylic acids, esters, aldehydes, ketones, and heterocyclic compounds (Kao et al., 2006). The release and accumulation of these synthetic nitriles within the environment poses a negative effect on all life forms and the environment (Egelkamp et al., 2019). Cyanide, is released from nitrile degradation is toxic to cells (both eukaryotes and bacterial cells). Neurologic, hepatic, cardiovascular and gastrointestinal disorders are experienced by humans and experimental animals as a result of nitrile exposure. The toxicity results mainly from the release of cyanide in the body.

Benzonitrile (C6H5C≡N) is a nitrile compound in which the hydrogen has been replaced by a phenyl group, making it a member of benzenes and a nitrile functional groups. Due to the vast application as a solvent and intermediate in the manufacturing of drugs, perfumes, dyes, rubber, textiles, agrochemicals, resins, and speciality lacquers, benzonitrile is considered the most used from synthetic nitriles. Benzonitriles are also used in the production of melamine, proactive coating, moulding resins, as an additive in jet fuel, nickel-plating, cotton bleaching baths, for drying acrylic fibre and in the preparation of benzoguanamine under alkali conditions. A major component in herbicides are benzonitriles (Mukram et al., 2015).

Severe environmental pollution is caused by cyanide occurrence when nitriles are

released into water bodies. Benzonitriles released as wastewater, from agricultural herbicides and chemical industries, leads to their increased distribution throughout the environment and requires detoxification (Mukram et al., 2015). The direct discharge of synthetic nitriles, which are toxic and some being carcinogenic and mutagenic, into the environment via wastewater discharge poses a health hazard (Kao et al., 2006). Health hazards caused by benzonitrile include decrease in motility, muscular force and methemoglobinemia. It is therefore imperative to remove benzonitrile from industrial effluents (Mukram et al., 2015). If these toxic compounds are not treated/decontaminated before entering the environment, environmental deterioration would result (Kao et al., 2006).

There are various methods, which are utilised to degrade these organic pollutants, and these include physical processes, chemical processes and biological processes. Physical process achieves degradation of organics with the use of photo electrodes, where separation is achieved through photo induced charges and formation of heterogeneous junction, or nanoparticles are used as a photocatalyst to degrade the organic pollutants. The use of chemicals to degrade organic pollutants is categorised as chemical processing and these include: chemical precipitation, neutralization, adsorption, disinfection (chlorine, ozone, ultraviolet light), and ion exchange (Samer, 2015). Several chemical and physical treatments are available to treat benzonitrile containing wastes, these include combustion and photo-oxidation. Due to the high operational costs, harsh reaction conditions and unwanted by products and secondary products of the above mentioned methods they are rendered as unfavourable (Mukram et al., 2015).

Living organisms such as microbes (bacteria and fungi) also possess the ability to degrade organic pollutants, and this type of degradation is categorised as biological degradation or bioremediation (Ratnakar et al., 2016). Bioremediation is the process whereby microorganisms are used to remove/degrade pollutants within a contaminated site, the process is conducted under controlled conditions. The main objective for bioremediation is to degrade the pollutant (organic pollutant) to concentrations below the permissible limits established by authorities (Ratnakar et al., 2016).

The advantages biological processes for decontamination of pollutants over physio-chemical processes include: harmful intermediates are not generated through biological

processes, bioremediation can be carried out on site thus reducing transportation risks and, bioprocesses are eco-friendly; economically sustainable and widely accepted by the general public (Ratnakar et al., 2016).

Therefore, a biological process is proposed as a remedial process for the detoxification of hazardous organic pollutants found in the environment.

This study focuses on the biological treatment method for nitriles, particularly synthetic nitrile such as benzonitrile, with emphasis on the biological degradation of benzonitrile. The study evaluates the utilization of microbial communities to grow in an environment with benzonitrile as sole source of energy (carbon and nitrogen) for bioprocessing purposes.

1.2 Problem Statement

Environmental pollution is a serious threat to all forms of life since these pollutants threaten the wellbeing of humans, animals and plants (Khan and Ghouri, 2011). Human activities such as industrial development; agricultural activities and technology advancement, urbanization and mining activities, contribute enormously to pollutants/contaminants being introduced to the environment. These mentioned human activities are major drivers of water, soil and air pollution that further affects the wellbeing of living organisms within the environment (van Huong et al., 2017).

The demand for chemically synthesized products through the use and production of nitriles poses a health hazard and environmental issue due to the improper disposal of nitrile containing waste. Benzonitrile is the most widespread synthetic nitrile used due to the vast application in industries. Therefore Benzonitrile contaminated waste requires adequate treatment before being discharged into the environment. There are chemical and physical treatment methods for nitrile waste, however these treatment methods are expensive and toxic by-products are formed which require further treatment. Hence bioremediation process which is an environmentally friendly, cost effective and does not produce toxic by-products is an effective alternative and preferred method of decontamination.

1.3 Hypothesis

It is hypothesised that microorganisms (bacteria) isolated from gold mine tailings are able to degrade organic compounds (nitriles).

1.4 Objectives

1.4.1 Overall objective

The aim of the study is to use microorganisms (bacteria) isolated from Gold (Au) mine tailings to biodegrade organic environmental contaminants of interest.

1.4.2 Specific Objectives

The objectives of the study are:

1. Isolate culturable microorganisms (bacteria) from gold mine tailings.
2. Identify microorganisms (bacteria) using molecular diagnostic tools.
3. Extract and analyse the organics from the gold mine tailings.
4. Bioremediate benzonitrile using the isolated microorganisms (bacteria).

CHAPTER 2: LITERATURE REVIEW

2.1 Gold (Au) mine tailing formation

South Africa is one of the largest gold mining nations in the world, Au mining began over a century ago (Rösner and Van Schalkwyk, 2000). In the earliest days of mining, mercury was used to separate the gold embedded within the rock. The processes of gold extraction was done by mixing of mercury with gold bearing ores forming an amalgam which would be burnt allowing for the vaporization of the mercury whilst the gold remained. Cyanide extraction of gold from the ore is the extraction procedure used by large scale gold mines as in South Africa. Extraction of gold with use of cyanide is achieved via two step/stage process namely extraction and recovery. The crushed rock/ore containing gold is mixed with cyanide which the gold dissolves into known as the extraction process. The gold is recovered from the first step by cementing the cyanide solution with zinc or use of activated carbon to absorb the gold (Fashola et al., 2016). The waste produced from mining activities is usually dumped to the environment with little pre-treatment.

2.2 Microbes in toxic environment

The chemical and physical properties of the tailings are determined by the nature of the ore, geochemistry, method used to extract the ore and the chemical processing of the ore to obtain the valuable material e.g. gold (Au). Key characteristics of gold (Au) mine tailings are the enhanced concentration levels of toxic metals such as mercury (Hg), cobalt (Co), zinc (Zn), copper (Cu), lead (Pb), nickel (Ni), cadmium (Cd) and arsenic (As) (Mendez et al., 2008). Poor aggregation, high hydraulic conductivity, fine texture and very limited cohesion ability are the physical properties of gold mine tailings. Mine tailings generally exhibit an acidic pH, although some tailings may be alkaline, and contain a very low organic content attributing to the nutritional deficient nature of mine tailings (Mendez and Maier, 2008).

The characteristics and nature of mine tailings including elevated concentrations of toxic metals and low/no presence of organics and micro-nutrients provides a stressful environment for microbes to live in. Toxic metals are not required for metabolomic function but can be accumulated by microbes (Gadd, 2010), which are limited to the

accessibility of the uptake of toxic metals (Fashola et al., 2016). The nature of the gold mine tailings environment exerts complex stresses for microbial communities to grow under. Thus giving rise to resistant microbes in the gold mine tailings environment. Factors that give rise to resistant microbes are changes in normal environmental conditions, types and levels of contaminations as well as geographical and geological origin of the environment. Resistance by microbes in contaminated environments are achieved by the development and adaptation to the detoxifying mechanisms by the microbes allowing for biosorption, bioaccumulation and biotransformation of toxic contaminants (Fashola et al., 2016).

The harsh environmental conditions and characteristics of mine tailings give rise to stress and resistant microbial communities (Mendez and Maier, 2008). Au mine tailings have been inhabited by both Gram negative and Gram positive bacteria such as *Pseudomonas, Aeromonas, Shewanella, Brevundimonas, Agrobacterium* and *Acinetobacter*, members of *Proteobacteria* phylum and *Bacillus*, *Serratia* and *Exiguobacterium* members of phylum *Firmicute* (Mendez and Maier, 2008).

2.3 Organic environmental contaminants

Environmental pollution caused by the use of chemical substances, such as hydrocarbons (aliphatic, aromatic, polycyclic aromatic hydrocarbons, BTEX (benzene, toluene, ethylbenzene, and xylenes), petroleum oil, chlorinated hydrocarbons, nitroaromatic compounds and organophosphorus compounds), solvents and pesticides. Since the introduction of the industrialization era where these organic contaminants have been produced at a large scale and disposed of into the environment (Megharaj et al., 2011). These organic contaminants are toxic and pose a threat to life forms. Organic contaminants are produced by many industries including: pharmaceuticals, personal care, veterinary products, pesticides, food additives, industrial compounds or by-products, and engineered nano-materials (Sorensen et al., 2015).

The increasing use of synthetic organic compounds by the agricultural, industrial and domestic setting (Sorensen et al., 2015), suggests that more organic contaminants will be produced. Potential sources of organic contaminants include: Leaks and spills from industrial processes, oil and chemical storage tanks leakages, pesticides improper

application, cleaners, oil and antifreeze disposed of into the environment, household

Wastes disposed into the environment, garbage dumps and landfills, leaks from pipelines containing oils or gases, and spillages occurring due to accidents during transportation of organic substances (Snousy et al., 2016).

Waste containing a chemical composition or other properties capable of causing illness, death or some harm to human and other forms of life when released into the environment or mismanaged can be defined as hazardous waste. Medical waste; household or municipal waste; agricultural waste and industrial waste are common sources of hazardous waste, which may lead to soil, air and water pollution (Snousy et al., 2016).

Table 2.1: Organic waste contaminants in the environment (adapted from Lamastra et al., 2016).

Compound Group	Compound class
Pharmaceuticals	Veterinary & human antibiotics; analgesics, anti- inflammatory & anti-histamine drugs; psychiatric drugs; lipid regulators; b-blockers & antihypertensives; X-ray contrasts; steroids & hormones; blood-viscosity affecting agents; antidiabetics; antidepressants; abuse drugs; stimulants
Personal care products	Fragrances; sun-screen agents; insect repellents; antiseptics, biocides; moth repellents; surfactants
Pesticides	Insecticides, fungicides, herbicides, nematocides, biocides
Food additives	Antioxidants, sweeteners, colorants
Manufacturing additives	Corrosion inhibitors; flame retardants; gas propellants, plasticisers, plastic additives; stain repellents; surfactants, antioxidants, solvents, paraffin
Biocides	Biocides

In this study we will be focusing on one environmental contaminant namely nitriles.

2.4 Nitrile contamination and the treatment of nitrile contamination

2.4.1 Nitrile contamination

Nitrile is an organic molecule which has a carbon double bonded to a nitrogen (–C=N) functional group. Nitriles can be found naturally in nature as well as being synthetically produced. Bacteria, fungi, plants,marine and terrestrial habitats are examples where nitriles are found in nature (Egelkamp et al., 2019; Egelkamp et al., 2017). Nitriles are also extensively used in the industrial sector to produce plastics, solvents and synthetic intermediates (Tanii, 2017), the pharmaceutical industry is also seeing an increase in nitrile containing pharmaceuticals (Fleming et al., 2010), some nitriles are also used in

herbicides formulations (Egelkamp et al., 2019). Man-made/synthetic nitriles that are released and accumulated within the environment has a negative impact on the environment and on all life forms (Egelkamp et al., 2019).

Nitrile toxicity originates from cyanide released during nitrile degradation. . By cytochrome P450 in eukaryotes. Cyanide, released from nitrile degradation, inhibits cytochrome c oxidases thus inhibiting the electron transport chain. A similar mode of action is observed in bacteria as cytochrome P450 and cytochrome c oxidase are present in many bacterial species (Egelkamp et al., 2019).

There are two enzymatic routes that facilitate nitrile (cyanide) detoxification and degradation. The first enzymatic route involves nitrilases (EC 3.5.5.1) belonging to the carbon-nitrogen hydrolase superfamily and harbour a catalytic triad consisting of glutamic acid, lysine, and cysteine. Nitriles are degraded directly to carboxylic acids and ammonia by nitrilases. In the second route nitriles are degraded to amides, catalysed by nitrile hydratase (NHases; EC 4.2.1.84), and subsequently hydrolysed to carboxylic acid and ammonia by amidases (EC 3.5.1.4) (Egelkamp et al., 2019; Egelkamp et al., 2017).

Neurologic, hepatic, cardiovascular and gastrointestinal disorders are experienced by humans and experimental animals as a result of nitrile exposure. The toxicity results mainly from the release of cyanide in the body.

2.4.2 Treatment of nitrile contamination

Nitrile compounds are extensively used in various industries such as synthesis of pharmaceuticals, drug intermediates, plastics, rubber, herbicides and pesticides. In 2001 the global industrial consumption of acetonitrile was 4 × 104 tons. Wastewaters from various industrial uses of nitriles often contain high contents of these compounds and therefore these wastewaters need to be effectively treated to minimize their effects on the environment and on living organisms before they are discharged. Treatment methods such as ozonation and photocatalytic oxidation have been applied to treat wastewaters containing nitriles (in order to degrade the nitriles), however these methods are of an economic burden and produce secondary pollutants that are toxic (Li et al., 2007).

Activities such as mining, mineral processing, electroplating and the plastic industry contribute significantly to cyanide (nitrile) contamination in the environment. Cyanide which is a nitrile is highly toxic to living organisms, thus wastewaters from these activities are contaminated with cyanide and need to be treated. Various chemical and physical methods have been deployed and utilized to degrade cyanide containing wastewaters, however these strategies have financial burdens, for example sludge is produced which requires further treatment and the by-products produced are hazardous (Mekuto et al., 2013).

2.4.3 Chemical treatment method

Most chemical treatment processes of nitrile/cyanide operate on the principle of converting cyanide into a less toxic compound through oxidation reactions (Botz et al., 2005). These chemical treatment methods include alkaline chlorination, sulphur dioxide and air process, hydrogen peroxide, Caro's acid process, ozonation. Factors that play a driving role in the feasibility of specific chemical treatment methods include the chemical composition of waste produced, reagents availability and hazardous nature of reagents, the volume and effluent quality (Botz et al., 2005).

2.4.3.1 Alkaline chlorination

Cyanide-destruction occurs in a two-step reaction, first cyanide is converted to cyanogen chloride (CNCl) and in the second step cyanogen chloride hydrolyses to yield cyanate.

$$Cl2 + CN- \rightarrow CNCl + Cl-$$
$$CNCl + 3H2O \rightarrow OCN- + Cl- + 2H+$$

In the presence of excess chlorine at a alkaline pH, cyanate is hydrolysed further to yield ammonia:

$$OCN- + 3H2O \rightarrow NH4+ + HCO3- + OH-$$

Alkaline chlorination is one of the oldest methods used for cyanide destruction, which was most widely applied method of cyanide treatment. The process can be expensive to operate due to high reagent usage. Alkaline chlorination process is carried out at a pH > 10.5 to ensure cyanogen chloride is rapidly hydrolysed to cyanate (Botz et al., 2005). The

disadvantages of alkaline chlorination are the addition of objectionable cations/anions to water, excess hypochlorine is toxic, chlorine may react with organics to form chlorinated compounds, toxic intermediates may arise from poor process controls (Parga et al., 2003).

2.4.3.2 Sulphur dioxide and air process

This process was developed and patented by the Inco Metals Company in 1984 in Canada and is known as the Inco process. A mixture of SO2 and air is used in the presence of a copper catalyst, under pH conditions of 8 – 10, to selectively oxidise bothe free and complex cyanide species to cyanate (Botz et al., 2005; Kuyucak and Akcil, 2013). Although the reagents are inexpensive, the process treats both aqueous solutions and Au mine waste sludge over a wide pH range (8 – 10). The drawbacks from this process include royalty payments, addition of sulfates to treated water and if precipitating ferrocyanides with copper the precipitate requires adequate disposal (Parga et al., 2003).

2.4.3.3 Hydrogen Peroxide

The hydrogen peroxide (H2O2) system has been used as early as 1980s by OK TEDI Mining Limited in New Guinea to treat cyanide. Hydrogen peroxide is used to oxidise free and weakly complex cyanide to cyanate , through a 1 step reaction, in the presence of a transition metal (Cu, Ag, V, Th) as a catalyst (Kuyucak and Akcil, 2013; Anning et al., 2019).

$$CN^- + H_2O_2 \rightarrow OCN + H_2O$$

The advantages of this process is that excess reagent decomposes to water and oxygen and the process is relatively simple to operate. However few disadvantages include the cost of reagents and adequate disposal of precipitate required if precipitating ferrocyanides with copper (Parga et al., 2003).

2.4.3.4 Caro's acid process

Peroxymonosulphuric acid (H2SO5), known as Caro's acid is produced from concentrated hydrogen peroxide and concentrated sulphuric acid. Due to its instability, Caro's acid is produced on-site and used immediately for cyanide detoxification with only

minimal intermediate storage. The reaction of Caro's acid with cyanide does not require a catalyst to oxidise cyanide to cyanate and typically completed within few min (Breuer et al., 2011).

2.4.3.5 Ozone

Ozone (O3) is a strong oxidant, capable of oxidising cyanide to cyanate, ammonia and nitrate. The reaction rate is rapid and limited by the rate at which ozone is absorbed into the solution. Ozone is relatively expensive to produce limiting its use for cyanide destruction (Botz et al., 2005).

2.4.3.6 Acidification-Volatilization-Regeneration (AVR)/Mills-Crowe process

Involves acidifying waste cyanide bearing solutions to a pH of 2 – 3 with H2SO4 or SO2, volatilizing the resulting HCN by air flushing and recovering the HCN by absorption in an alkaline solution (NaOH or Ca(OH)2). The Mills-Crowe or AVR process has successfully been applied to several mining sites (Kuyucak and Akcil, 2013).

2.5 Benzonitrile

2.5.1 Background on Benzonitrile

Synthetic nitriles (Man-made) are widely used in the manufacturing of feedstock, extractants, solvents, drug intermediates, pharmaceuticals, and pesticides. They also form important intermediates in the organic synthesis of amines, amides, amidines, carboxylic acids, esters, aldehydes, ketones, and heterocyclic compounds (Kao et al., 2006).

Benzonitrile (C6H5C≡N) is considered the most toxic from amongst all synthetic nitriles due to its wide application as a solvent and intermediate in the manufacturing of drugs, perfumes, dyes, rubber, textiles, agrochemicals, resins, and speciality lacquers. Benzonitrile is also used in the production of melamine, proactive coating, moulding resins, as an additive in jet fuel, nickel-plating, cotton, bleaching baths, for drying acrylic fibre and in the preparation of benzoguanamine under alkali conditions. Herbicides such as dichlorobenil (2,6-dichlorobenzonitrile), bromoxynil (3,5-diromo-4-hydroxybenzonitrile), and ioxynil (4-hydroxy-3,5-diiodobenzonitrile) utilizes benzonitrile as a major component (Mukram et al., 2015).

Nitriles released into water bodies result in letting cyanide, which persist into soil and surface water causing severe environmental pollution. Benzonitrile utilization as agricultural herbicides and chemical industries release of benzonitriles as wastewater leads to their increase in distribution throughout the environment and therefore requires its detoxification (Mukram et al., 2015). The discharge of these synthetic nitriles directly into the environment via wastewater is an alarm for possible health hazard, pointing to the toxicity these compounds have some being carcinogenic and mutagenic. An environmental problem in the future is waiting to arise if these toxic compounds in waste are not treated (Kao et al., 2006).

Health hazards caused by benzonitrile include decrease in motility, muscular force and methemoglobinemia. Leading to the imperative need to remove benzonitriles from the industrial effluents (Mukram et al., 2015).

2.5.2 Treatment of Benzonitriles

There are chemical and physical approaches for treating benzonitriles, however the financial implications render these methods too expensive (Kao et al., 2006). Several chemical and physical treatments are available to treat benzonitrile containing wastes, these include combustion and photo-oxidation. Due to the high operational costs, harsh reaction conditions and unwanted by products and secondary products of the above mentioned methods they are rendered as unfavourable (Mukram et al., 2015). The use of microorganisms to bioremediate nitriles is preferred over chemical and physical treatments, as biological methods are cost effective and environmentally friendly (Mukram et al., 2015).

Microbial hydrolysis of nitriles makes use of two major enzymatic pathways. In the first pathway, nitrile hydratase (EC 4.2.1.84) which catalyses nitriles to their amides, which are then hydrolysed to their carboxylic acid and ammonia by amidase (EC 3.5.1.4). The second pathway, nitriles undergo direct hydrolysis to their carboxylic acids and ammonia by nitrilases (EC 3.5.5.1). Aliphatic nitriles, follows the first pathway during bioremediation, are broken down to their acids and ammonia via the formation of amides. Whereas aromatic nitriles such as benzonitrile have shown to be directly converted to

carboxylic acid and ammonia by nitrilase, following the second enzymatic pathway of microbial degradation/hydrolysis (Mukram et al., 2015).

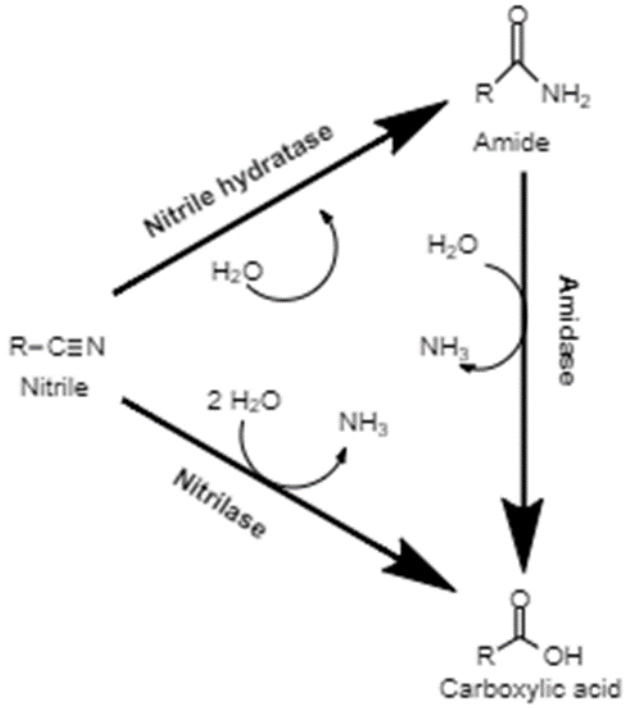


Figure 2. 1: hydrolysis of nitrile yielding ammonia and carboxylic acid (Sahu et al., 2019).

2.6 Biological degradation of nitriles: Enzymes, mechanism of action and bacteria containing nitrile degrading enzymes

2.6.1.1 Nitrilase function and structure

Enzymes that catalyses the conversion of nitriles to their corresponding acid and ammonia are termed nitrilases (Thuku et al., 2009). Nitrilases are members of a superfamily whose general function is to catalyse non-peptide carbon-nitrogen bond hydrolysis. There are 13 branches within the nitrilase superfamily. Nitrilases belongs to branch 1 of the superfamily, together with structurally and functionally related cyanide hydratase and cyanide dehydratase enzymes. Nitrilases, like most superfamily members, contains a αββα structural fold and a catalytic triad Cys-Glu-Lys active site which is flanked by distinctive conserved signature sequences within a branch, but differ amongst member branches. It is noted that most protein dimerization occurs in many of these enzymes together with the absence of prosthetic groups or metal cofactors. A large helical linear aggregates between 2 – 26 subunits formed by nitrilase enzyme dimers, with substrate

specificity-dependent activation shown by most enzymes (Chhiba-Govindjee et al., 2019).

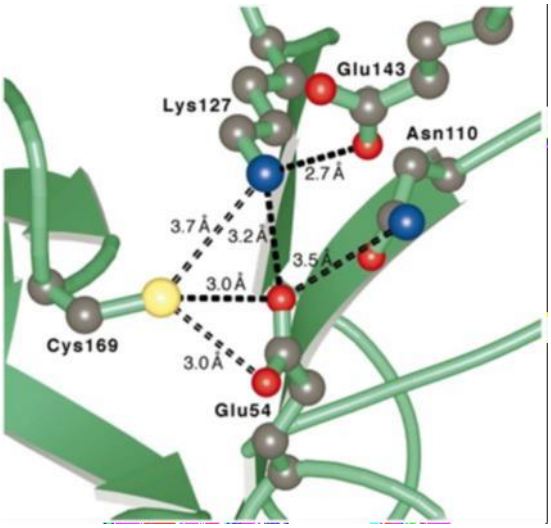


Figure 2. 2: Presumed active site of nitrilase, indicating related residues of Glu54, Lys127 and Cys169 forming functional catalytic triad within the nitrilase superfamily (Pace et al., 2000; Gong et al., 2012).

2.6.1.2 Mechanism of action

A catalytic triad composed of cysteine, glutamate, and lysine residues are the active site of nitrilases. A second glutamate is also important for the enzymatic reaction. The importance of cysteine residue within the catalytic triad through detection of a covalently bond reaction intermediate, identified as thioimidate or acylenzyme, and by the generation of inactive enzyme variants after the exchange of the cysteine against other amino acid residues. Therefore, it is accepted that nitriles are initially bond to nitrilases via a cysteine residue in form of a thioimidate, which is finally hydrolysed to a tetrahedral intermediate and subsequently deaminated to an acylenzyme, which is finally hydrolysed to the carboxylic acid. Glutamate residue of the catalytic triad of this reaction acts as a general base catalyst and that the lysine stabilises the tetrahedral intermediate (Stolz et al., 2019).

Nitrilases contains a catalytic triad containing Cys-Glu-Lys making up the enzymes active site. Nitrile hydrolysis reaction catalysed by nitrilase takes place via a tetrahedral intermediate. Jiang et al., (2017) postulate that the tetrahedral intermediate can be

hydrolysed through two pathways (Fig 2.3) (Jiang et al., 2017). In pathway A, ammonia is eliminated from the tetrahedral intermediate to form corresponding acid as the final product, this is a major pathway followed by nitrilase (Jiang et al., 2017). Nitrilase reaction mechanism has been postulated that the nitrile carbon bears a fractional positive charge which is subjected to nucleophilic attack by one of the two SH groups on the nitrilase, forming an imine. The resulting imine is then hydrolysed to corresponding ketone while having an ammonia (NH3) as a by- product. Acyl-enzyme is then hydrolysed by the addition of water (H2O), and finally liberates the carboxylic acid along with the regenerated enzyme (Gong et al., 2012).

Pathway B (Figure 2.3) eliminates the formal thiol of Cys to produce an amide, the amide production is a completely different mechanism in pathway be compared to that of Nitrile hydratase (NHase),

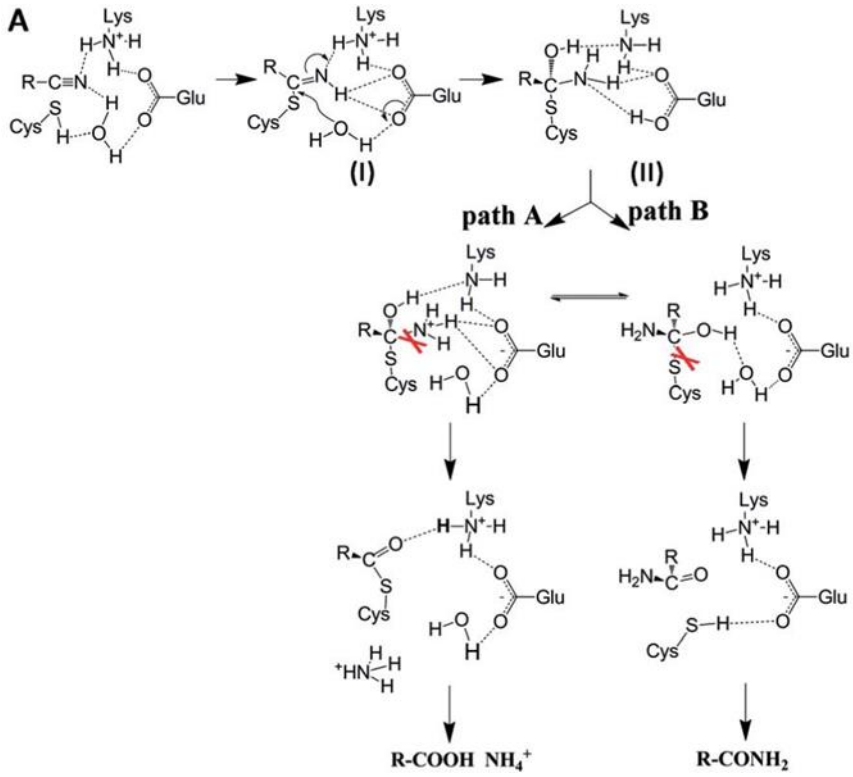


Figure 2. 3: Proposed mechanism of Nitrilase indicating pathway A and B (Jiang et al., 2017).

2.6.1.3 Bacteria containing Nitrilase activity

The Pseudomonas genus isolated from soil was the first bacterial strain to hydrolyse

ricinine nitrile group, the bacterial identification was based primarily on morphological characteristics. Several bacteria were reported to contain nitrilase activity, these include: *Rhodococcus, Nocardia, Acinetobacter, Alcaligenes, Pseudomonas,* and *Corynebacteriam* (Gong et al., 2012).

Aromatic nitrilases are active against aromatic and heteroaromatic substrates such as benzonitriles and 3-cyanopyridine, and are generally less active against aliphatic nitriles. Bacteria containing these aromatic nitrilases include *R. rhodochrous* ATCC 39484, *R. rhodochrous* J1, *Rhodococcus sp*. NDB1165, *Rhodococcus rhodochrous (Nocardia)* NCIB11216/NCIMB 11216, *Nocardia globerula* NHB-2, *Rhodobacter sphaeroides* LHS-305, *Acinetobacter sp*. AK226, and *Bacillus pallidus* DAC521 (Chhiba-Govindjee et al., 2019).

Table 2. 2: Reported bacteria with nitrilase activity extracted from (Adapted from Gong et al., 2012).

Bacteria	Molecular weight (kDa)	Optimum pH / temperature (°C)	Stability pH / temperature (°C)	Substrate specificity
Pseudomonas sp.	-	-	7.4-8.8 / -	N-Methyl/ethyl-3-cyano-4methoxy-2-pyridone
Nocardia sp. NCIB 11216	560	8 / -	-	Aromatic nitrile
Brevibacterium strain R312	-	7 / 35	- / below 30	-
Pseudomonas sp. 13	1,000	9 / 55	7-11 / below 60	β-Cyano-L-alanine
Acinetobaeter sp. APN	-	-	-	α-Aminonitriles
R. rhodochrous J1	78	7.6 / 45	- / 20-50	Aliphatic, aromatic nitriles
Comamonas testosteroni sp.	-	-	-	Aliphatic nitriles
A. faecalis JM3	275	7.5 / 45	7-8 / 20-50	Arylacetonitriles
Acinetobacter sp. AK226	580	8 / 50	5.8-8 / below 60	Aliphatic, heterocyclic nitriles
A. faecalis ATCC 8750	460	7.5 / 40-45	6.5-8 / below 50	Arylacetonitriles
R. rhodochrous PA-3445		7.5 / 35	- / below 35	Aromatic, aliphatic nitriles
R. rhodochrous K22	650	5.5 / 50	- / below 55	Aliphatic nitriles
Klebsiella ozaenae	37	9.2 / 35	-	Bromoxynil
Bacillus pallidus Dac521	600	7.6 / 65	6-9 / below 65	Aromatic nitriles
R. rhodochrous NCIMB 11216	45.8	8 / 30	-	Aromatic nitriles
P. fluorescens DSM 7155	130	9 / 55	-	Arylacetonitriles
N. globerula NHB-2	-	-	-	Aromatic, unsaturated aliphatic nitriles
P. putida	412	7 / 40	6.5-8 / below 50	Arylacetonitriles
Pyrococcus abyssi GE5	60	7.4 / 80	4.5–8.5 / 60-90	Aliphatic dinitriles
Acidovorax facilis 72W	570	- / 65	5-10 / below 60	Aliphatic dinitriles
Rhodococcus sp. NDB1165	-	8 / 45	- / below 50	Aromatic and unsaturated aliphatic nitriles

Table 2.2 continued…

Bacteria	kDa	Optimum pH / Temperature (°C)	Stability pH / temperature (°C)	Substrate specificity
Bradyrhizobium japonicum USDA110	455	-	-	Mandelonitrile, phenylacetonitrile
Halomonas nitrilicus sp. nov.	-	-	-	Arylaliphatic nitriles
Bacillus subtilis ZJB-063	-	-	-	Arylacetonitriles
Alcaligenes sp. ECU0401	376	8 / 40	- / below 50	Aliphatic and aromatic nitriles
P. fluorescens Pf-5	138	7 / 45	- / below 65	Dinitriles
Streptomyces sp. MTCC 7546	-	-	-	Aliphatic nitriles
Arthrobacter nitroguajacolicus ZJUTB06-99	-	6.5 / 40	- / below 50	Aliphatic and aromatic nitriles
R. erythropolis ZJB-0910	-	7.5 / 30	- / below 30	β-Hydroxy aliphatic nitrile
Geobacillus pallidus RAPc8	600	-	-	Aromatic nitriles
A. faecalis ZJUTB10	-	7.7–8.5 / 35	- / below 35	-
A. faecalis MTCC 10757	-	8 / 35	-	Aliphatic and aromatic nitriles

2.6.2 Nitrile Hydratase (NHase)

2.6.2.1 Nitrile Hydratase (NHase) function and structure

Nitriles are hydrolysed to their corresponding amides in a reaction catalysed by nitrile hydratase (NHase), the reaction is usually followed by an amidase catalysed step converting the amide to the corresponding carboxylic acid and ammonia (Foerstner et al., 2008).

NHase are metalloenzymes which contain either a non-heme Fe(III) or non-corrin Co(III) in their active sites and are typically α2β2-heterotetramers. Each α-subunit in all NHase known contains a highly homologous amino acid sequence (C*X*YCSC*X*) that forms the metal-binding site. Threonine and tyrosine are contained in the active site amino acid sequence of cobolt-type NHase –C(T/S)YCS(Y/T)-, whereas in the active site amino acid sequence of iron-type NHase contains serine or threonine. The trivalent metal ion of both iron and cobolt type NHase is six-coordinate with three cysteine and two amide nitrogens

making up the remainder of the ligands. Two of the active site cysteine residues are modified to cysteinsulfinic acid (-SO2H) and cysteinsulfenic acid (-SOH) post-transitionally, yielding a metal coordination geometry termed “claw setting”, activation of NHase only occurs if this Cys oxidation process occurs. Nitric oxide (NO) binds in place of a metal-coordinated water/hydroxide molecule in iron-type NHase, which can be photoactivated, whereas cobolt-type NHase does not bind NO. Theoretically the carbon-nitogen bond in the coordinated amide of both NHase has double bond character, suggesting an imidometal bond best represents it (Mitra and Holz, 2007).

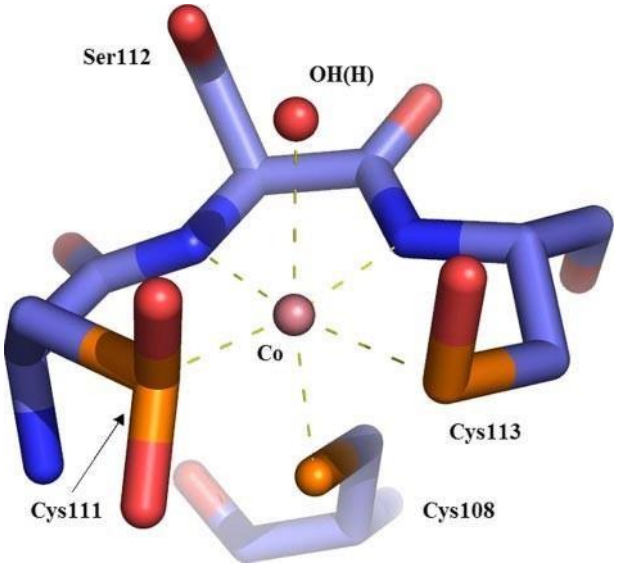


Figure 2. 4: Active site of NHase from P. themophila. The active site contains a trivalent metal ion (Co) which is six-coordinate, with three cysteine sulfurs, two amide nitrogen and a water molecule. Post-transitionally Cys111 is modified to cysteine sulfinic acid and Cys113 is modified to cysteine sulfenic acid, yielding “claw setting” of the metal activate site (Mitra and Holz, 2007).

2.6.2.2 Reaction Mechanism of NHase

The full reaction mechanism of NHase remains elusive. The proposed mechanism based on large quantum-mechanical active site models. The proposed mechanism of NHase identifies Cys-SO- as a nucleophile, performing a direct attack on the metal coordinated nitrile. Forming a cyclic intermediate, which is subsequently cleaved through attack of the axial cysteine on the sulfenate, forming a disulphide bond. Nitrile hydration occurs without direct involvement of water molecule in this mechanism. Subsequent water-mediated disulphide cleavage regenerates the active site (Hopmann, 2014).

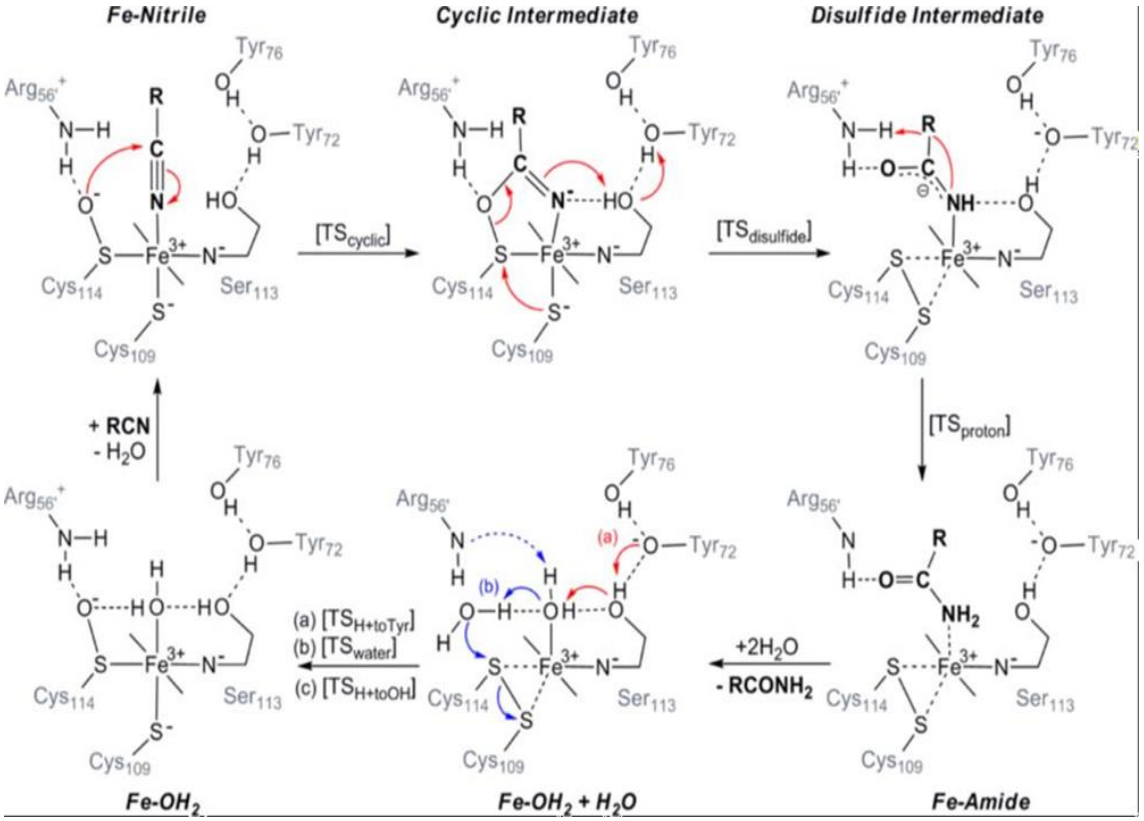


Figure 2. 5: Reaction mechanism of NHase proposed by Hopmann, (2014).

The attack of Cys114-SO on the coordinated nitrile forms a cyclic intermediate. Cleavage of the cyclic intermediate occurs through attack of Cys109 on the Sulphur atom. Cys109-S S - Cys114 disulfide formation promotes cleavage of the latter to give the amide. Active site regeneration occurs through attack of water on the disulfide Cys114-SO- attacks the coordinated nitrile to form a cyclic intermediate. The cyclic intermediate is converted to the amide, this is achieved through cleavage of cyclic intermediate by attack of Cys109 on the sulfur atom. Resulting in the formation of a Cys109-Cys114 disulfide bond, with the simultaneous cleavage of Cys114-substrate bond and a proton transfer from Tyr72 via Ser113 to the substrate. The amide product incorporates the oxygen atom of Cys114. The disulphide is formed from a thiolate and sulfonate. Following the formation of disulphide intermediate, the substrate nitrogen abstracts a proton from Arg56. Following displacement of amide, sequential steps of proton transfer occurs to Tyr72, attack of water on the disulphide, and proton to Arg56, to regenerate the active site. (Hopmann, 2014).

2.6.2.3 Bacteria containing NHase activity

NHase are found in species belonging to phyla Proteobacteria, Actionobacteria,

Cyanobacteria and Firmicutes, their habitats range from soil, costal marine sediments and deep sea sediments to geothermal environments (Foerstner et al., 2008).

2.6.3 Amidases

2.6.3.1 Amidase function and structure

The conversion of amides to carboxylic acids and ammonia, during nitrile breakdown, is catalysed by amidase. Amidases (EC 3.5.1.4) are classified into two groups, based on their amino acid sequence and structural similarities, namely the nitrilase superfamily amidase and the amidase signature family. Amidases belonging to the nitrilase superfamily exists as a homotetrameric or homohexameric complexes, which contains a catalytic triad consisting of a conserved Glu-Cys-Lys in the catalytic centre. The amidase signature family enzymes form homodimeric or homooctameric complexes, containing a conserved sequence consisting of 130 amino acids, with a catalytic triad consisting of Ser-Ser-Lys in the catalytic centre (Ohtaki et al., 2010).

2.6.3.3 Reaction mechanism of amidases

The reaction mechanism utilized by amidase can be described as a ping-pong bi-bi mechanism. Amidase binds to the amide substrate, ammonia is released. A thioester intermediate is formed at the cysteine, water binds as the second substrate and the release of carboxylic acid product occurs (Weber et al., 2013).

It has been generally accepted that the catalytic reaction mechanism of amidase, whereby Cys166 acts as nucleophile, Glu59 acts as a general base catalyst and Lys134 stabilizes the tetrahedral intermediate. However Kimani et al., (2007), suggested that Glu59 is not a candidate for the general base catalyst as it is buried behind the acyl intermediate based on geometry of the active site of G.pallidus RAOc8 amidase. Thereby suggesting a second glutamate residue (Glu142) is better positioned to act as a general base catalyst (Kimani et al., 2007).

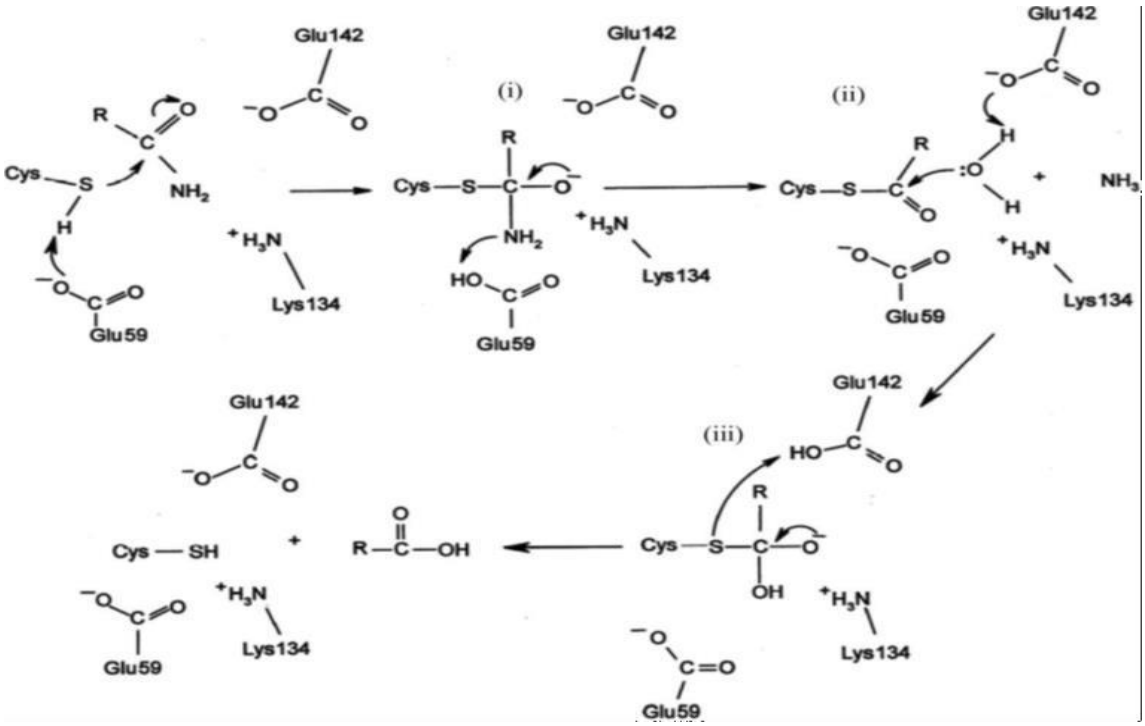


Figure 2. 6: Proposed reaction mechanism of amidase (isolated from G. pallidus RAPc8). Cys166 acts as a nucleophile. (i) The reaction mechanism proposed by Kimani et al., (2007) proposes that Glu142 acts as a general base catalyst, thereby enhancing the nucleophilicity of the water which hydrolyses the acyl intermediate (ii). Glu142 is positioned at a distance pf 3.7 Å from the acyl carbon and has the necessary stereoelectronic considerations and is suggested because Glu59 is buried beneath the acyl group. Lys134 stabilizes the tetrahedral intermediate. The source of the proton in step (iii) is speculative, as the distance between Cys166 S and the carboxylate O atom of Glu142 is 5.3 Å (Kimani et al., 2007).

2.6.3.3 Bacteria containing amidase activity

Amidases have been reported to exist within all kingdoms within the world, these enzymes can be found in bacteria; fungi; plants and animals. The genera of bacteria that have been reported to contain amidases are: *Corynebacterium*, *Mycobacterium*, *Pseudomonas*, *Bacillus*, *Micrococcus*, *Brevibacterium*, *Nocardia*, *Streptomyces*, *Blastobacter*, *Arthrobacter*, *Alcaligenes*, *Helicobacter*, *Lactobacillus*, and *Methylophilus* (Fournand and Arnaud, 2001)

2.7 Bioremediation technology: Background, principles, role of bacteria and, the advantages of bioremediation

2.7.1 Background on bioremediation technology

Microorganisms are extensively spread through the biosphere due to their metabolic activity and ability to easily grow under a vast range of environmental conditions. Exploiting their nutritional versatility, microorganisms can be utilized for biodegrading of environmental pollutants. Bioremediation can be defined as the use of microorganisms to biodegrade pollutants. Bioremediation process is achieved through the ability of the microorganism to convert, modify and utilize toxic pollutants to obtain energy and biomass. Microorganism(s) under the bioremedial process conduct themselves in a well organised procedure whereby pollutants or toxins are broken down or transformed to less or non-toxic compounds. Biological agents such as bacteria, archaea and fungi are known as bioremediators, as they are able to conduct bioremedial activities thereby cleaning or breaking down toxins and contaminants. Pollutants and harmful toxins accumulated in the environments can be tackled with the use of microorganisms. The application of bioremediation is achieved through the process by which microorganisms utilize the toxins or pollutants as an energy and carbon source. In turn breaking down the toxins and cleaning up contaminated environmental sites. The advantages of bioremediation are cost effectiveness (cheaper treatment method) and also an environmentally friendly approach as compared to physical and chemical treatments currently used and promises valuable genetic material to combat environmental issues (Thakkar and Vincent, 2021).

2.7.2 Principle of bioremediation

The task of contaminant destruction is achieved through microorganisms due to the enzymes which they possess allowing them to utilize environmental contaminants as a source of food and energy. Bioremediation is aimed at encouraging the microorganism(s) to work together and degrade or detoxify hazardous environmental contaminants by providing optimal levels of nutrients and other chemicals essential to their metabolism. Enzymes mediate all metabolic reactions, enzymes are grouped based on the reactions they mediate namely: oxidoreductases, hydrolases, lyases, transferases, isomerases and ligases. Enzymes have a wide degradation capacity mainly to their specific and non-specific substrate affinities. Bioremediation effectiveness is achieved when enzymes of the microorganism enzymatically attack the pollutant and convert it to a harmless product.

As this goal of bioremediation can only be achieved where environmental conditions permit the growth and activity of microorganisms, therefore manipulation of these controlling parameters are done to achieve microbial growth and activity which will allow for pollutant degradation at optimal rates meaning optimised bioremediation processes (Thakkar and Vincent, 2021).

2.7.3 Role of bacteria in bioremediation

Bioremediation success depends on the ability of microorganisms to utilize contaminants of interest as nutrient or energy source (thus degrading the contaminant). Single microorganisms generally metabolise a limited range of substrates, whereas a mixture of microorganisms with the ability to metabolize a certain substrate is more advantageous for bioremediation purposes as the mixed microorganism population has a broader enzymatic capacity to increase the rate and extent of extent of biodegrading the contaminant (Patowary et al., 2016). Microbial consortia consist of mixed bacterial/microorganism cultures, which are utilized for bioremediation works and are more advantageous than single pure cultures. Interactions amongst associated members of the consortium may lead to complete degradation of the contaminant. Survival of microorganism in contaminated site/medium and desired degradation potential are characteristics by which microorganisms are selected for a desired microbial consortium (Divya et al., 2016). Environmental degradation processes are generally carried out by a consortium of microbes rather than by a single microbe (Divya et al., 2016). Wastewater treatment and crude oil degradation are examples of the successful utilization of microbial consortia to treat contaminated sites (Divya et al., 2016; Patowary et al., 2016).

2.7.4 Advantages of bioremediation

Bioremediation technologies exploits the natural process whereby microorganisms are able to grow in contaminated environment utilizing/degrading the contaminant as a nutrient and energy source. As the contaminant is depleted the population of microorganisms decrease. Resulting in residues of the treatment with carbon dioxide, water and cell biomass as harmless product of bioremediation (Thakkar and Vincent, 2021). Due to the natural process being carried out, bioremediation is an eco-friendly method for contamination clean-ups (Divya et al., 2016; Thakkar and Vincent, 2021). Bioremediation is preferred method of decontamination over physical and chemical

methods such as photo-oxidation and combustion, because they are costly operations, they require harsh reaction conditions and they produce unwanted by products and secondary pollutants. The cost effectiveness and the environment friendliness aspect of biological methods makes bioremediation technologies ideal for decontamination strategies (Mukram et al., 2015). Bioremediation can be carried out on site of contamination, eliminating the transport costs and potential health and environment threats may arise from the risk of transporting hazardous chemicals. Decontaminating as site is less labour intensive as the natural role of microorganisms and the environment is being used in the bioremediation process. The use of bioremediation technologies is environmentally friendly and sustainable; also harmful chemicals are not used in this process of decontamination. A site decontaminated can potentially be reused as bioremediation method is a nonintrusive method of decontamination (Thakkar and Vincent, 2021).

CHAPTER 3: RESEARCH METHODOLOGY

3.1 Site description and sample collection

The site of interest for this study was the Grootvlei Proprietary mine dump situated in the eastern region of the East Rand Basin within the Blesbokspruit catchment in the Gauteng province of South Africa. Grootvlei had been actively been mined for gold for over 80 years prior to its forced closure in 2011. The Global Positioning System (GPS) coordinates for the site are: -26.218639, 28.485722.

Gold (Au) mine tailings were sampled from the gold mine dump. Samples were collected, as mentioned by Okereafor et al., (2020), from four different spots on the site at intervals of 100 m for fair representation of the area. Samples were collected using a disinfected auger to drill to a depth of 20 cm. The process was repeated at the different spots before transferring each tailing sample into designated bags. The samples were transported for further analysis for microbes and organic compounds.

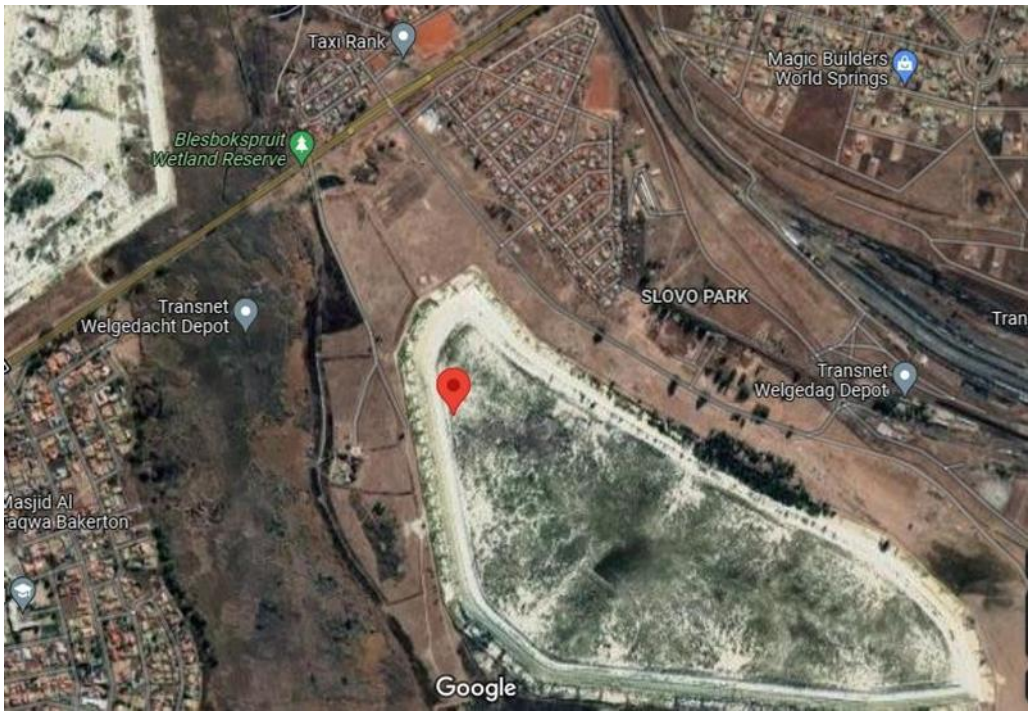


Figure 3. 1: A satellite view of the collection site of Au mine tailings, GPS coordinates - 26.218639, 28.485722. (Image extracted from Google).

3.2 Isolation and Identification of microbes

Microorganisms were cultured from Au mine tailing samples and single colonies of cultured microorganisms were isolated using microbiological techniques. Isolated microorganism were further investigated with molecular diagnostic tools to identify the single colonies present in Au mine tailings.

Gold (Au) mine tailings sample (1g) were submerged in sterile distilled water and vortexed for homogeneity. 100 µl of the sample were spread plated across the Luria Broth (LB) agar plates (for bacterial growth). The LB agar plates were incubated at 37 °C overnight where the developed single colonies present on the LB agar plates were further streaked onto fresh LB agar plates for purification of the isolates such that pure isolates were isolated.

Molecular techniques such as DNA extraction and Polymerase Chain Reaction (PCR) were used to obtain the DNA sequence of isolated single bacterial colonies for identification.

Pure single colony growth of each microbe was achieved, and the organisms were sent to Inqaba Biotech® (Pretoria, South Africa) for DNA sequencing and identification. The 16S rDNA of bacterial colonies was sequenced for the identification of bacterial species. DNA was extracted using ZR Fungal/Bacterial DNA kit (Zymo research). DreamTaq DNA polymerase (Thermo Scientific) and the 16S forward and reverse primers (16S-27F with 5’ to 3’ AGAGTTTGATCMTGGCTCAG and 16S-1492R with 5’ to 3’ sequence CGGTTACCTTGTTACGACTT) sequence was used to amplify the 16S rDNA target region through Polymerase Chain Reaction (PCR). PCR products were ran through an agarose gel and thereafter, they were gel extracted (Zymogen Research, Zymogen Gel DNA recovery kit), and sequenced in forward and reverse directions on the ABI PRISM 3500xl Genetic Analyser. Purified products (Zymogen Research, ZR-96 DNA Sequencing Clean-Up Kit) were analysed using CLC bio Main Workbench 7 followed by a Blast search.

The DNA sequences obtained was used to construct a phylogenetic tree to identify microorganisms based on their DNA sequence. For identification of the bacterial colonies, the 16SrDNA gene sequences (query) were compared to sequences of Gene

Bank database, five relatives were obtained for each of the query sequence with an identity of > 99%. Accession numbers were obtained by submitting the query sequences to NCBI GenBank. A phylogenetic tree was constructed by aligning the query sequence and the 5 relatives using ClastalW multiple sequence alignment on BioEdit (version 7.0.5.3) and MAFFT – a multiple alignment programme online version. MEGAX software (version 10.0.5) was used to construct the phylogenetic tree using the neighbor-joining method with 1000 bootstraps.

3.3 Extraction and identification of organic molecules/compounds found in the gold (Au) mine tailings.

3.3.1 Extraction of organic molecules/compounds from gold (Au) mine tailings.

Organic compounds were extracted from Au mine tailing sample via a liquid-solid extraction. Organic solvents, hexane, dichloromethane (DCM) and ethyl acetate (EtOH), were used as organic extractants. The rationale behind these organic solvents was based on availability and the ability for these organic solvents to extract organic compounds by means of like attracts like.

The samples were mixed with organic solvents and agitated, the mixtures were decanted onto Graphman filter paper with the organic compounds collected in the solvent in a separate beaker (Moldoveanu and David, 2015; Chung and Alexander, 1998). Organic compounds extracted from samples were concentrated on a rotor vapour which allowed the solvent to evaporate. The concentrated samples were prepared for organic analysis via liquid chromatography.

The concentrated samples were suspended in 2 ml of mobile phase A (running solvent of LC instrument). Samples were sonicated for 2 min and filtered with a 0.45 µm syringe filter, thereafter 100 µl of the filtered sample was added into a glass LC vial and 900 µl of running solvent (mobile phase A) was added to the vial.

3.3.2 Identification of non-volatile organic compounds via LC-QTOF-MS

Identification of non-volatile organic compounds, present in Au mine tailings, was achieved through Liquid chromatography time of flight mass spectrometry. Liquid chromatography was used for separation of compounds within the sample. Mass

spectrometry was used to identify compounds based on their mass/charge (m/z) ratio. Non-volatile organic compounds were analysed using the liquid chromatography time of flight mass spectrometer. The above mentioned samples, in section 3.3.1, prepared for LC were analysed for non-volatile compounds.

Analysis of gold (Au) mine tailings samples for identification of organics was done on a LC-QTOF-MS system with a Dionex UltiMate 3000 UHPLC (Thermo Scientific, Darmstadt, Germany) coupled to a Compact™ QTOF (Bruker Daltonics, Bremen, Germany) that used an electrospray ionization (ESI) interface, with a modified method by Hoffmann et al., (2014); Tapfuma et al., (2019). An injection volume of 5 µl of the sample was used in the system for chromatographic separation of analytes/compounds in reverse phase ultra-high-performance liquid chromatography (RP-UHPLC) through a Raptor ARC-18 column with dimensions of 2.7 µm (particle size), 2.1 mm (internal diameter), 100 mm (length) and 90 Å (pore size) (Restek, Bellefonte, USA). The mobile phase was composed of solvent A consisting of 0.1 % formic acid in H2O (v/v) and solvent B consisting of 0.1 % formic acid in acetonitrile. A gradient elution from 5% A to 95% B was used to separate analytes from the samples.

The parameters of the ESI was set at: a set capillary voltage at 4.5 kV; end plate offset at –500 V; dry heater temperature at 220 °C; dry gas flow rate at 2.5 L/min and nebulizer gas pressure at 1.8 Bar. Mass spectra operated in the positive ion mode, with the scan range of 50 – 1300 m/z. Instrumentation operation, control and data acquisition was done using HyStar software version 2.10 (Thermo Scientific, Darmstadt, Germany). Spectral data processing was performed using Bruker Compass DataAnalysis software version 4.3 (Bruker Daltonics, Bremen, Germany). MetFrag Handler version 2.1 software (GitHub, California, USA) was used to characterize the resulting fragment spectra by linking to three compound databases, namely PubChem, ChemSpider and KEGG (Tapfuma et al., 2019).

Data obtained from LC-QTOF-MS was analysed and compounds were identified. Organic compounds with the highest peak intensity were considered as compounds of environmental interest.

3.3.3 Identification of volatile organic compounds via GC-TOF-MS

Identification of volatile compounds, present in Au mine tailings, was achieved through GC-TOF-MS. Gas chromatography was used for the separation of volatile compounds within the sample. Mass spectrometry was used to identify the volatile organic compounds based on their m/z ratio.

Analysis of gold (Au) mine tailing samples for identification of volatile organics was done utilizing a GCxGC/MS system. The system used was an Aligent 7890 gas chromatography system with a LECO GCxGC option, coupled to a Time-of-flight (TOF) detector interface coupled to a MS detector.

Chromatographic separation occurred through the primary and secondary columns on the GCxGC system, with BPX-5 as the primary column with dimensions of 27.878 m (length) x 250.00 µm (diameter) x 0.25 µm (film thickness); and Rxi-17Si as the secondary column with dimensions of 1.027 m (length) x 250.00 µm (diameter) x 0.25 µm (film thickness). Front injector was used which operated in split mode, with helium (He) as the carrier gas with a flow rate of 1.40 ml/min. The inlet temperature was set at 300°C. The column temperature was set at 50 °C for 0.5 min with an increased gradient temperature of 10 °C to reach a targeted temperature of 305 °C was reached and temperature stayed constant for 5 min. The secondary column temperature was set with + 5 °C relative to the primary column.

The mass spectrometer (MS) operated to identify compounds with a mass range of 45 – 800. Electron energy of 70 V was used with the ion source temperature was set at 250 °C. Data processing utilized mainlib and replib as libraries to identify molecules with m/z ratio of 45 – 800.

Data obtained from LC-QTOF-MS was analysed and compounds were identified. Organic compounds with environmental interest were selected for biodegradation studies. Aminobenzonitrile was selected as a compound of interest, which was further investigated in biodegradation studies by the identified bacteria.

3.4 Preliminary biodegradation trials

3.4.1 Preparation of microorganisms inoculums

Inoculum for preliminary biodegradation trials were prepared by inoculating single colonies (from glycerol stocks) of identified bacterial species into Nutrient Broth media. The inoculated bacterial species were incubated at 30 °C for 16 – 24 hrs. and agitated at 180 rpm in a shaking incubator. Mature cultures were used at a 10 % (v/v) of the total volume for the preliminary biodegradation trials.

3.4.2 Biodegradation trials

Biodegradation trials were conducted in minimal media (M.M) without the presence of a nitrogen source. The M.M contained (g/L): K2HPO4 (4.3); KH2PO4 (3.4); MgCl2.6H2O (0.4); and Sodium Acetate (0.1). The compound of interest, 3-aminobenzonitrile (Fluka, Germany), at a concentration of 100 mg/L was added to the M.M. The inoculum prepared above was inoculated into the M.M containing 3-aminobenzonitirle at a final concentration of 10 % (v/v) and incubated at 30 °C for 72 hrs. and agitated at 180 rpm (Mekuto et al., 2016). A total of five bacterial species were tested in the biodegradation trials.

3.4.3 Analysis of free ammonium as a product of nitrile degradation

Samples (2 mL) were obtained from biodegradation trials and centrifuged at 16 000 xg for 15 minutes. The centrifuged supernatant was analysed for ammonium concentration using Merck ammonium ($NH4^{+}$) (00683) test kit. A Merck Spectroquant Nova 60 was used to quantify the residual concentration of ammonium, as mentioned by Mekuto et al., (2013).

3.5 Process optimisation

3.5.1 Formation of bacterial consortium

A bacterial consortium was formed by combining single bacterial colonies which yielded a positive result in the ammonium test previously. This was accomplished by initially inoculating single bacterial colonies from glycerol stock into Luria-Bertani (LB) broth media, which were then incubated at 30 °C for 16 - 24 hrs. with agitation at 180 rpm in a shaking incubator. After achieving growth of each bacterial single colony strain separately, bacterial colonies were pooled together to form a consortium. The consortium

was formed by adding 1 ml of each inoculum (bacterial strains grown overnight) into LB media. The consortium was incubated at 30 °C for 16 - 24 hrs. with agitation at 180 rpm in a shaking incubator.

3.5.2 Response surface methodology: central composite design

Response surface methodology (RSM) is a modelling technique that uses a combination of mathematical and statistical methods to evaluate the relationship between variables and the outcomes/results. The central composite design (CCD) was used in this study and a 20-run experimental plan was generated to obtain optimal parameters for temperature, pH and, substrate (aminobenzonitrile) concentration. For this study the optimum parameters were investigated with temperature ranging from 25 °C to 35 °C; pH ranging from 7 to 10 and the initial concentration of aminobenzonitrile concentration ranging from 50 to 150 mg/L. The Design Expert ® software (version 7.0.0, Stat-Ease Inc., Minneapolis, USA) was used to set up RSM; CCD and statistical analysis of optimisation experiments.

The bacterial consortium formed was used for the optimisation process. Cultures of the consortium were grown up at conditions determined by CCD (table 3.2) for a period of 72 hrs., and the inoculum concentration was 10 % (v/v) of the total volume (Mekuto et al., 2016).

Table 3. 1: The various media variables under investigation through the CCD for optimisation within their ranges.

Variables	Units	Low Actual	High Actual	Low Coded	High Coded	Mean
Temperature	°C	25.00	35.00	-1.000	1.000	30.000
pH		7.00	10.00	-1.000	1.000	8.500
Substrate Concentration	mg/L	50.00	150.00	-1.000	1.000	100.000

Table 3. 2: Central composite design for the three variables.

Run	Temperature (°C)	pH	Substrate concentration (mg/L)
1	21.59	8.50	100.00
2	35.00	10.00	50.00
3	30.00	8.50	100.00
4	35.00	7.00	50.00
5	30.00	8.50	15.91
6	30.00	8.50	100.00
7	25.00	7.00	150.00
8	25.00	7.00	50.00
9	30.00	11.02	100.00
10	30.00	8.50	100.00
11	30.00	8.50	184.09
12	30.00	8.50	100.00
13	30.00	8.50	100.00
14	30.00	8.50	100.00
15	30.00	5.98	100.00
16	25.00	10.00	150.00
17	25.00	10.00	50.00
18	35.00	10.00	150.00
19	35.00	7.00	150.00
20	38.41	8.50	100.00

3.5.3 Analytical analysis

Samples were taken prior to the bioremediation tests as well as after the completion of the tests for each experiment. Each Sample was syringe filtered using a 0.45 µm filter. Qualitative and quantitative analysis were done using GC/MS.

3.5.3.1 Qualitative analysis using GC/MS

Qualitative analysis enabled the identification of compounds present before and after the bioremediation test runs.

GC/MS analysis (qualitative) was carried out on a GC system (Agilent 7820A) coupled to a quadrupole detector interface which was coupled to a MS detector (Agilent 5977B), used for identification of compounds, with a modified method of (Mukram et al., 2015). Chromatographic separation was carried out on an Aligent 7820A GC system equipped with a split/splitless injector and an Aligent 122-7032UI: DB Wax UI column (length 30 m, inner diameter 0.25 mm, 0.25 µm film) (Restek). A sample of 2 µl was injected into the injector under splitless injection mode, with the inlet temperature set at 280 °C. The column temperature range from 60 – 250 °C, with a gradient temperature increase of 15 °C/min and an isothermal period at 250 °C for 10 - 15 min, helium (He) gas was used as a carrier gas at a flow rate of 1 ml/min. The mass detector operated in electron impact ionization (EI) mode, with scanning range set at 10-500 m/z. The interface (quadrupole) and source (mass spectrometer) were 150 – 230 °C. MassHunter software (version 10.0.0368) was used for data processing and identification of compound peaks based of their m/z ratio and fragmented ions, NIST14 and Chemdecron libraries were used for the identification of compounds.

3.5.3.2 Quantitative analysis of aminobenzonitrile using GC/MS

Quantitative analysis was carried out on the GC/MS which enabled for the quantification of aminobenzonitrile to be determined after bioremediation tests were conducted.

For Quantitative analysis, samples were prepared to be used as standards with varying concentrations of aminobenzonitrile ranging from 20 – 500 mg/L in M.M. The standard samples were filtered and ran on GC/MS with the same conditions mentioned previously

(section 3.5.3.1). The standard samples were used to construct a standard curve using MassHunter Quantitative analysis software (version 10.0.368). The initial and final samples for the bioremediation tests were extrapolated automatically from the standard curve graph on the MassHunter Quantitative software.

Biological removal efficiency (BRE) was determined as follows:

$$BRE\ (\%) = (Ci-Cf)/Ci \times 100 \qquad \text{Eq.3.1}$$

Where the Biological removal efficiency is calculated by the difference in aminobenzonitrile concentration (Ci is the initial concentration of aminobenzonitrile and Cf is the final concentration of aminobenzonitrile), divided by the Ci (initial concentration). Then multiplied by 100.

The quantitative data achieved was used further down in the optimization studies.

3.5.4 Statistical analysis

Statistical analysis was done using analysis of variance (ANOVA), which included factors such as Fisher's F- test (overall model significance), the correlation coefficient (R) and determination of coefficient (R2). ANOVA assess the significance of the independent variables and their interactions. The F-test value indicates the overall significance of the model, the R was used to determine the strength of relationship between variables and, R2 was used to determine the proportion of the variance in the dependent variable that is predicted from the independent variable (indicates the level of variation in the data set).

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Isolation and identification of Bacteria from Au mine tailings

4.1.1 Introduction

Bacterial species capable of growing under harsh environmental conditions are resistant to the stressful environment in which they grow. The nature and characteristics of Au mine tailings includes high levels of toxic metals and low/no presence of nutrients providing an extremely stressful environment for microbes to survival and grow. Resilient bacterial species were isolated from Au mine tailings and pure colonies identified with use of molecular biological tools.

4.1.2 Objectives

- Isolate culturable microorganisms (bacteria) from gold mine tailings.
- Identify microorganisms (bacteria) using molecular diagnostic tools.

4.1.3 Results and Discussion

Due to the characteristics and environment of Au mine tailings which contains high levels of toxic metals and low nutrients making Au mine tailings a stressful environment for microbial growth (Gadd, 2010; Fashola et al., 2016). Resistant microbial communities are able to inhabit such harsh living conditions due to them containing coping mechanisms. Development and adaptation of detoxifying mechanisms by microbes allows these microorganisms to bioabsorb, bioaccumulate and biotransform toxic compounds within the environment. Based on the nature of Au mine tailings giving rise to resistant bacterial communities, this study focused on bacterial species isolated from Au mine tailings to be utilized for the degradation of organic compounds (nitriles) further down the study.

The extraction of genomic DNA from these isolated and amplification via PCR, depicted in Figure 7.1 (appendices), allowed for identification of the isolated bacterial species on a phylogenetic tree (Figure 4.1). A total number of five (5) bacterial colonies were isolated from Au mine tailings (Table 1). The isolated bacterial species identified as *Paenibacillus shunpengii (P. shunpengii), Bacillus pumilus (B. pumilus), Bacillus*

safensis (B. safensis), Bacillus thuringiesis (B. thuringiesis) and *Bacillus cereus (B. cereus)* shown in table 4.1 and figure 4.1. The isolated bacterial species belonged to one of two genus *Paenibacillus* or *Bacillus.* A total of four (4) species belonging to the *Bacillus* genus while one (1) belongs to *Paenibacillus* genus. The dominant genus to inhabit Au mine tailings is *Bacillus* followed by *Paenibacillus*. Previous studies by Mendez and Maier, (2008) have identified *Bacillus* from mine tailings amongst other genus which included *Pseudomonas, Aeromonas, Shewanella, Brevundimonas, Agrobacterium* and *Acinetobacter*.

Bacterial organisms were isolated from the Au mine tailing samples and the genomic DNA was extracted from the isolated bacterial samples. The 16S rDNA target region was amplified via PCR and the PCR products of 16S rDNA region that were ran on agarose gel is depicted in Figure 7.1 (Apendices).

The PCR products were extracted from the agarose gel and sequenced in the forward and reverse directions. Purified sequencing products were compared to sequences of GeneBank database, with sequences with > 99% identity, in order to characterise and identify bacterial colonies. The sequences were submitted to NCBI GenBank for accession numbers (see Table 4.1). The DNA sequences (query) obtained from sequencing were aligned with five relatives for each sequence using ClastalW multiple alignment on BioEdit (version 7.0.5.3) and MAFFT to align the sequences. Construction of the phylogenetic tree of the bacterial samples (Figure 4.1) utilized computational software MEGAX software (version 10.0.5) via neighbour-joining method with 1000 bootstraps done on the aligned sequences.

Table 4. 1: Accession numbers assigned to bacterial samples isolated from Au mine tailings.

NCBI GenBank Accession Number	Sequence ID	Organism name
MW300681	BRB_M1	*Paenibacillus shunpengii*
MW300682	BRB_M2	*Bacillus pumilus*
MW300683	BRB_M3	*Bacillus safensis*
MW300684	BRB_M4	*Bacillus thuringiesis*
MW300685	BRB_M5	*Bacillus cereus*

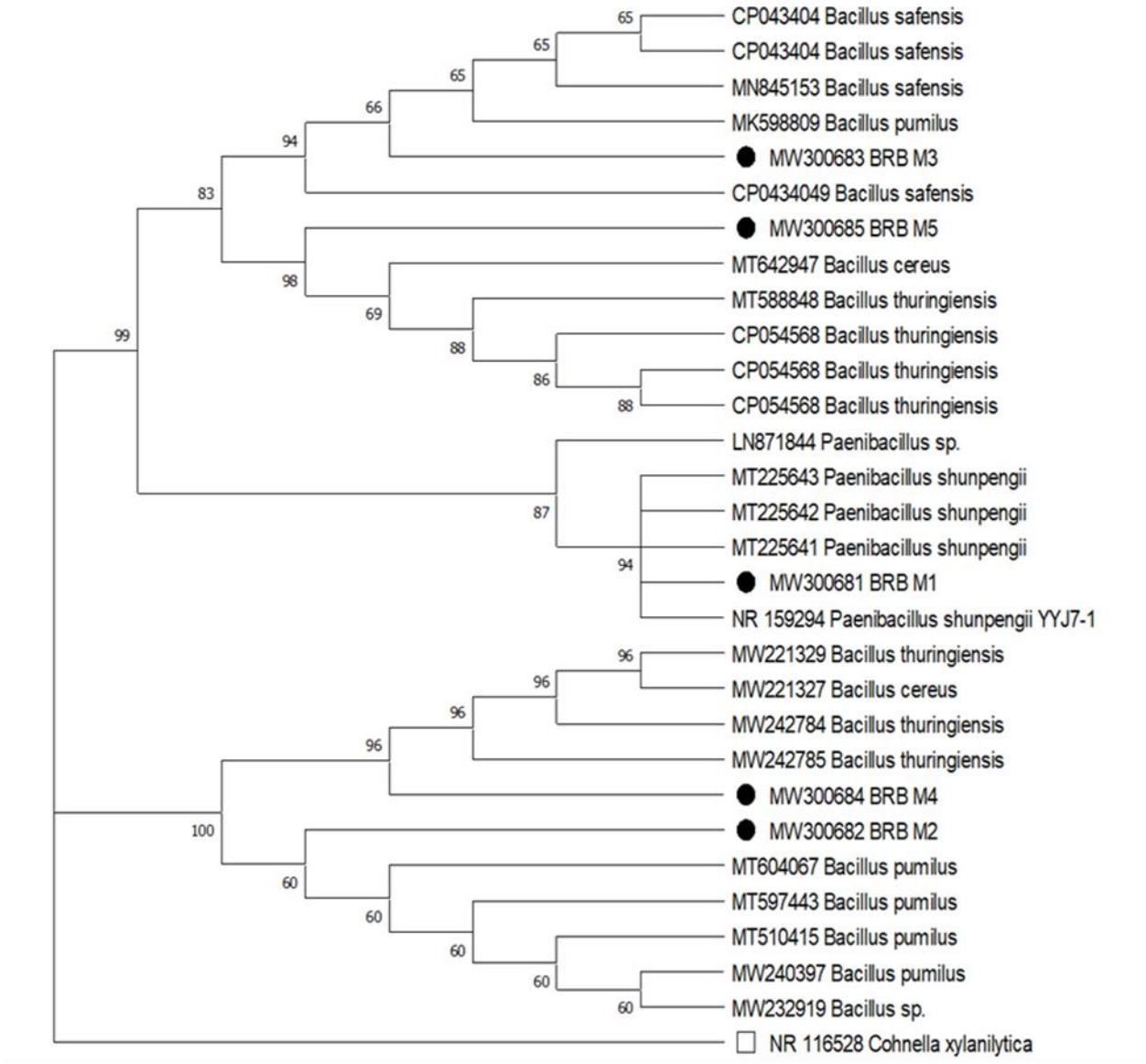


Figure 4. 1: The phylogenetic tree of the isolated organisms depicting the evolutionary relationships of taxa of bacterial species isolated from Au mine tailing samples.

The Neighbor-Joining method was used to infer evolutionary history of the samples to bacterial species (Saitou and Nei, 1987). The principle of the neighbor-joining method is to find pairs of operational taxonomic units (OTUs [=neighbors]) that minimizes the total branch length at stage of clustering of OTUs starting with a starlike tree. The branch lengths as well as topology of a parsimonious tree can quickly be obtained by using this method (Saitou and Nei, 1987). Figure 4.1 indicates the optimal phylogenetic tree indicating evolutionary relationship of bacterial species taxa with the sum of branch

length = 1.94495332. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The phylogenetic tree is drawn to scale (figure 4.1), with branch length in the same units as those of evolutionary distances used to infer the tree. Maximum Composite Likelihood method was used to compute the evolutionary distances (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Involved in this analysis were 31 nucleotide sequences. Codon positions $1^{st} + 2^{nd} + 3^{rd}$ + Noncoding were included. All ambiguous positions were removed for each sequence pair via pairwise deletion option. A total of 416 positions were used in the final dataset. MEGA X software was utilized to conduct evolutionary analysis (Kumar et al., 2018).

Based on the evolutionary taxa of bacterial species seen in the phylogenetic tree, BRB M1, was identified as *Paenibacillus shunpengii (P. shunpengii)* with a 94% on the bootstrap test; BRB M2 as *Bacillus pumilus (B. pumilus)* with 60% bootstrap test; BRB M3 as *Bacillus safensis (B. safensis)* with 65% bootstrap test; BRB M4 as *Bacillus thuringiesis (B. thuringiesis)*, with 96%, and BRB M5, identifies as *Bacillus cereus (B. cereus)*, with 69% bootstrapping.

4.1.4 Summary

The identified microorganisms obtained from Au mine tailings possess resilient traits in order to survive and grow in the type of setting isolated and identified from. A total of 5 bacterial species were isolated from Au mine tailings. The isolated microorganisms from Au mine tailings belonged to the *Bacillus* and *Paenibacillus* genus, with 4 species belonging to *Bacillus* and 1 belongs to *Paenibacillus* genus. The microorganisms isolated from Au mine tailings identified as *P. shunpengii*, *B. pumilus*, *B. safensis*, *B. thuringiesis* and *B. cereus*.

4.2 Extraction and Identification of Organic molecules present in Au Mine Tailings

4.2.1 Introduction

Since the introduction of the industrialization era, chemical wastes disposal into the environment have become a common phenomenon. These disposal practices lead to environmental pollution. Organic contaminants are toxic and threaten all life forms. Mine tailings are formed from the waste produced from mining activities which are usually dumped onto the environment with little or no pre-treatment.

Organic molecules present in Au mine tailings are of an interest as they contribute to environmental pollution and threatening life forms. This emanated from a previous study which detected a heavy presence of organics in a wetland that is adjacent to the location of the mine tailings, hence, this study was aimed at identifying the organic molecules present in Au mine tailings which are of environmental concern.

4.2.2 Objectives

1. Extract organics from the Au mine tailings using solvent extraction and analyse the extracted organics using liquid and gas chromatography techniques.

4.2.3 Results and discussion

4.2.3.1 Organic compounds detected via LC-QTOF-MS

Untargeted screening of non-volatile organic compounds extracted from Au mine tailings for the purpose of identifying organic compounds of environmental concern. Solid-liquid extraction was performed using three (3) organic solvents such as dichloromethane (DCM), ethyl acetate and hexane to validate the extraction and recover substantial organic compounds. Separation of non-volatile compounds was achieved using Liquid chromatography (LC) and identification occurred through mass spectrometer (MS). LC-MS is commonly used for determination of polar, non-volatile, analytes in environmental samples (Pitarch et al., 2016).

The reverse phase liquid chromatography enabled separation of organic compounds based on their polarity. Non-polar compounds bound to the stationary phase while polar compounds travel with the polar mobile phase and eluted out of the column. The

chromatograms depicts the elution profiles of compounds extracted with DCM (Figure 4.2), ethyl acetate (Figure 4.3) and hexane (Figure 4.4). Eluted compounds were detected using mass spectrometry, which detects compounds based on their mass/charge (*m/z*) ratios. The compounds eluted from the three extraction solvents were compared to the control sample, which contained only dH2O, to eliminate compounds found in the control. Organic compounds eluted out from the column are shown in table 4.2 (DCM extraction), table 4.3 (ethyl acetate extraction) and table 4.4 (hexane extraction) indicating the compounds' Retention time (RT), *m/z*, name, molecular formula and structure. A total of 9 compounds were identified using LC-QTOF-MS with 4 compounds being extracted by DCM, only 1 compound was extracted using ethyl acetate and 5 compounds extracted using hexane. The RT indicates the time it took for the compound to be eluted out from the column while the MS detector detects the *m/z* of the eluted compound. Based on the technique deployed for separation of compounds polar compounds are eluted out of the column first obtaining a lower RT. The mass of a compound is determined by the MS detector, which detects smaller compounds first.

Non-volatile organic compounds were analysed on the liquid chromatography time of flight mass spectrometer. The chromatograms (figures 4.2; 4.3 and 4.4) shows compounds eluted from the LC column which were extracted with DCM, ethyl acetate and hexane. Tables (4.2; 4.3 and 4.4) indicates the compounds identified via LC-MS with DCM, ethyl acetate and hexane as extraction solutions.

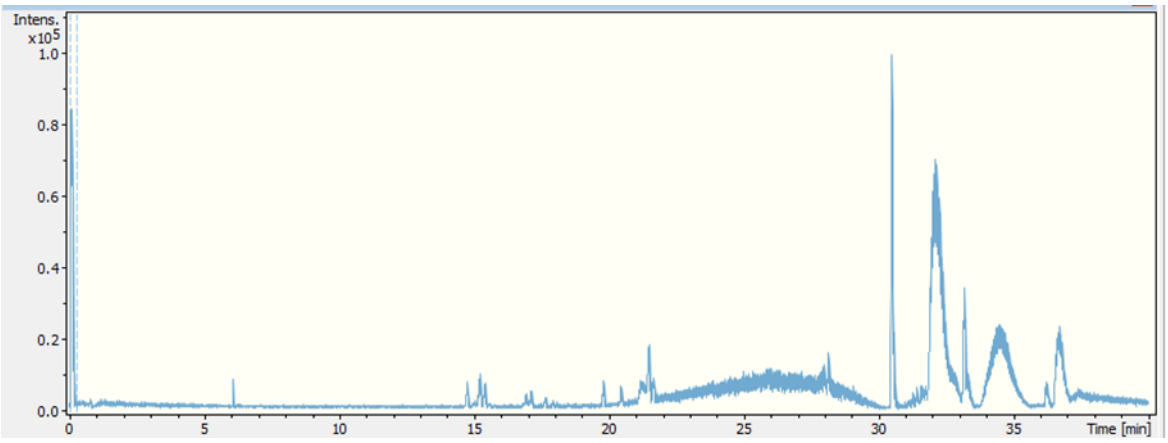


Figure 4. 2: Chromatogram of Au mine tailing sample with DCM as extractant showing compounds eluted out from the LC column.

Table 4. 2: Non Volatile compounds extracted from Au mine tailings using DCM.

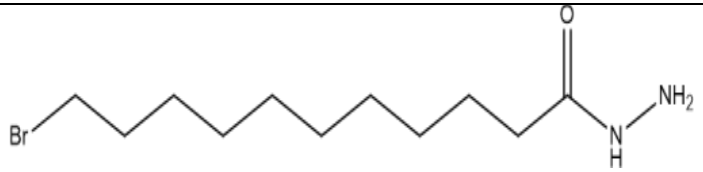
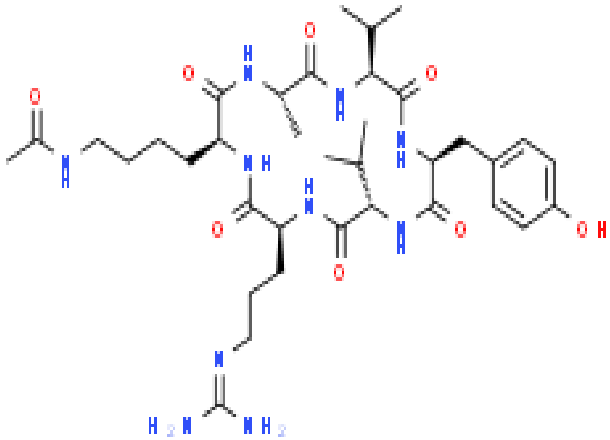
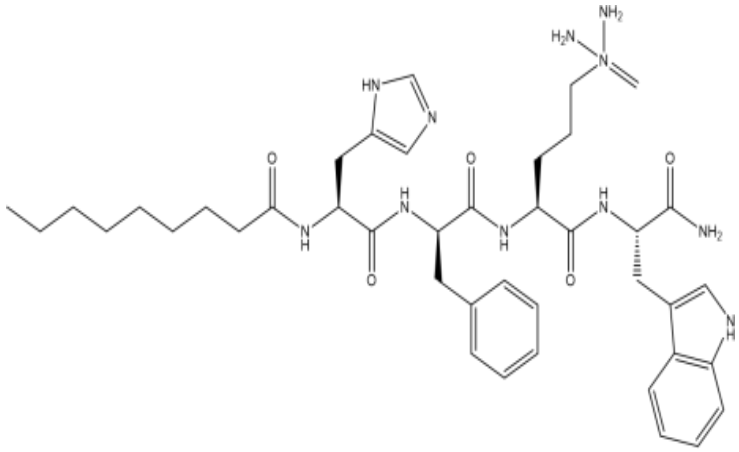
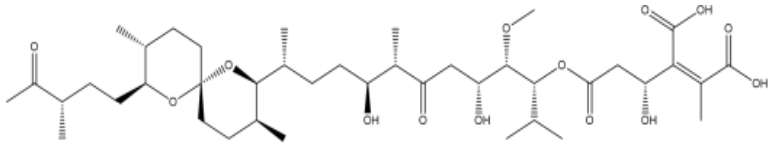
RT (min)	*m/z*	Compound Name	Molecular formula and database ID	Structure
21.19	279	11-Bromoundecanehydrazide	C11H23BrN2O Chem:Spider: 34660443	
25.79	759	Cyclo[L-alanyl-L-valyl-L-tyrosyl-L-valyl-N5-(diaminomethylene)-L-ornithyl-N6-acetyl-L-lysyl]	C36H58N10O8 ChemSpider: 8756346	

Table 4.2 continued …

RT (min)	m/z	Compound Name	Molecular formula and database ID	Structure
23.38	784	N-[(2S)-1-[[(2R)-1-[[(2S)-1-[[(2S)-1-amino-3-(1H-indol-3-yl)-1-oxopropan-2-yl]amino]-5-(diaminomethylideneamino)-1-oxopentan-2-yl]amino]- 1-oxo-3-phenylpropan-2-yl]amino]-3-(1H-imidazol-5-yl)-1-oxoprop-2-yl]nonanamide	C41H57N11O5 PubChem: 11158573	
23.38	785	Tautomycin diacid	C41H68O14 PubChem: 44451975	

The compounds identified are of complex structure and contain more than one functional groups. 11-Bromoundecanehydrazide, contains alkyl halide and hydrazide functional groups. Compounds Cyclo[L-alanyl-L-valyl-L-tyrosyl-L-valyl-N5-(diaminomethylene)-L-ornithyl-N6-acetyl-L-lysyl] and N-[(2S)-1-[[(2R)-1-[[(2S)-1-[[(2S)-1-amino-3-(1H-indol-3-yl)-1-oxopropan-2-yl]amino]-5-(diaminomethylideneamino)-1-oxopentan-2-yl]amino]-1-oxo-3-phenylpropan-2-yl]amino]-3-(1H-imidazol-5-yl)-1-oxoprop-2-yl]nonanamide, both have benzene ring structures and carboxamide functional groups. Tautomycin diacid was also identified and Ju et al., (2009) reported that tautomycin is a potent and selective inhibitor of protein phosphatase 1, which is diversely distributed throughout all cell tissue and regulates many diverse pathways from cell progression to carbohydrate metabolism (Kelker et al., 2009). Tautomycin is a molecular toxin which modulates protein phosphatase 1 activity (Kelker et al., 2009).

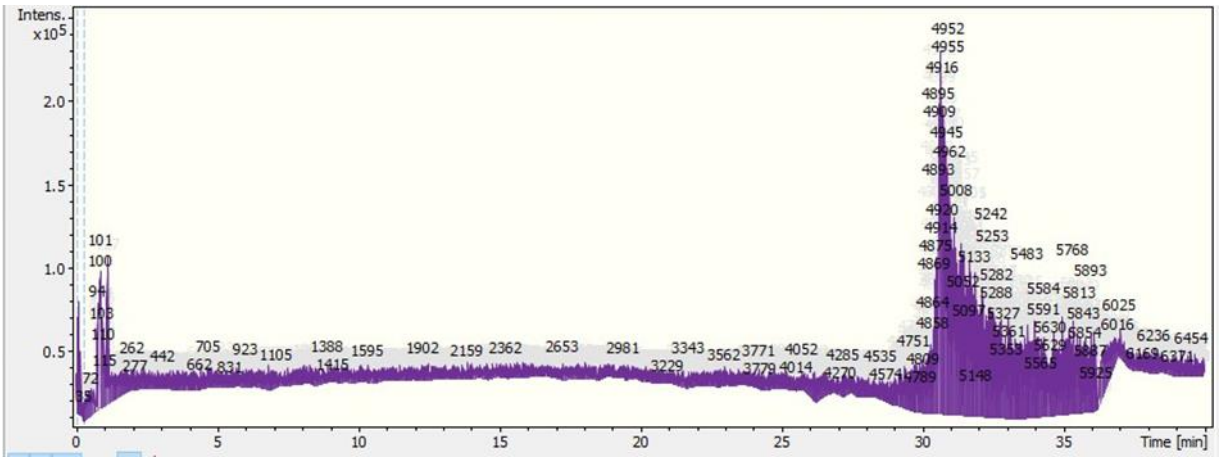


Figure 4. 3: Chromatogram of Au mine tailing with ethyl acetate as an extractant showing. analytes/compounds eluted from the LC column.

Table 4. 3: Non-volatile compound extracted from Au mine tailings using ethyl acetate as an extractant.

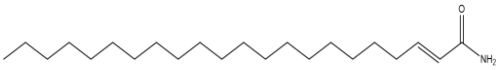
RT (min)	*m/z*	Compound Name	Molecular formula and database ID	Structure
24.41	338	2-Docosenamide	$C_{22}H_{43}NO$ Chemspider: 57561664	

Figure 4.3 indicates the chromatogram for separation of compounds extracted from Au mine tailings with ethyl acetate used as the extracting solvent. MS detected 2-docosenamide with m/z of 338, and RT of 24.41 min.

Although the chromatogram is congested, whereas table 4.3indicates only 1 compound being identified. This is due to the compounds being cross- referenced with a control sample and therefore, the peaks detected in the control were eliminated from the actual sample. Indicating that majority of the compounds detected was bleeding compounds on the column and not extracted from the sample. Hence, 2-docosenamide was the only compound extracted using ethyl acetate.

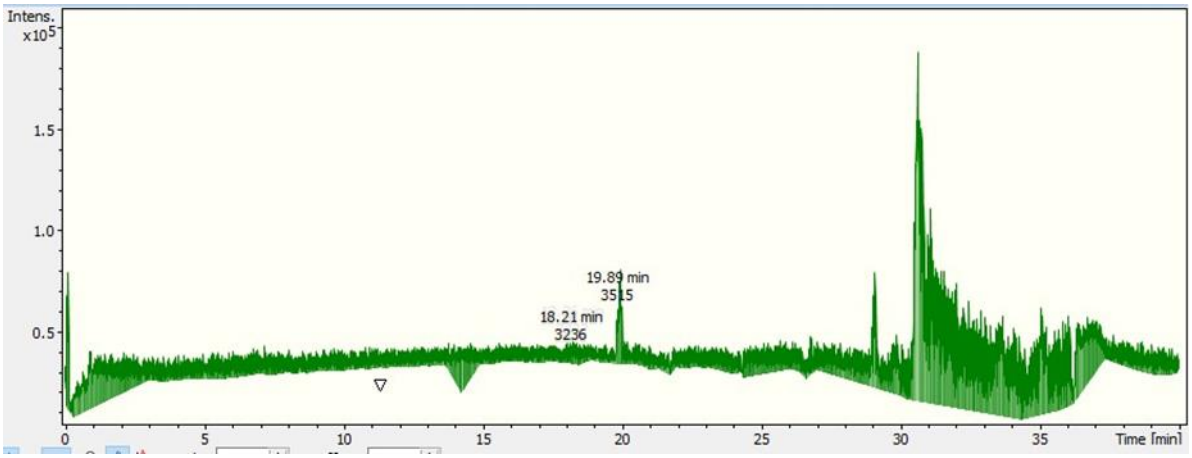


Figure 4. 4: Chromatogram of Au mine tailing with hexane as an extractant showing compounds eluted out from the LC column.

Table 4. 4: Non-volatile compounds extracted from Au mine tailings using hexane as an extractant.

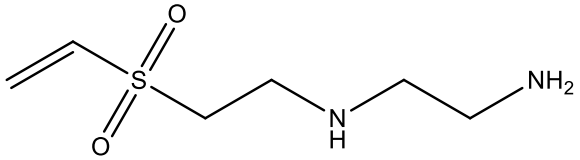
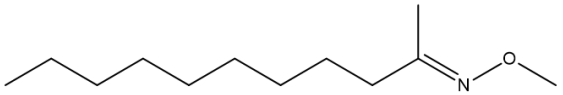
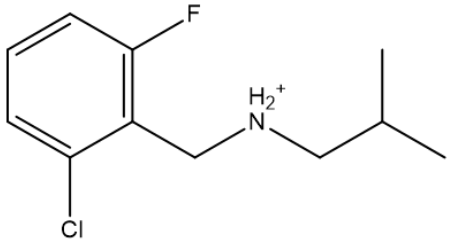
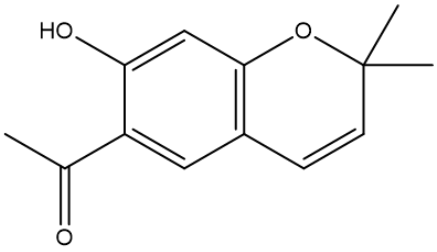
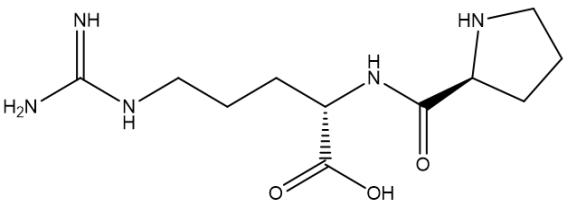
RT (min)	*m/z*	Compound Name	Molecular formula and database ID	Structure
5.92	179	N-(2-ethenylsulfonylethyl)ethane-1,2-diamine	$C_6H_{14}N_2O_2S$ PubChem: 19745002	
19.98	200	N-methoxyundecan-2-imine	$C_{12}H_{25}NO$ PubChem: 9602199	
18.24	217	N-(2-Chloro-6-fluorobenzyl)-2-methyl-1-propanaminium	$C_{11}H_{16}ClFN$ ChemSpider:6118127	
18.28	219	Eupatoriochromene; 1-(7-Hydroxy-2,2-dimethyl-2H-1-benzopyran-6-yl)-ethanone	$C_{13}H_{14}O_3$ KEGG: C09006	
19.19	272	Pro-Arg	$C_{11}H_{21}N_5O_3$ ChemSpider: 133096	

Figure 4.4 indicates the chromatogram of the non-volatile organic compounds found in Au mine tailings through a solid/solvent extraction using hexane. Table 4.4 indicates the compounds identified from the chromatogram and mass spectrometer with the retention times (RT), mass to charge ratio, compound name, molecular formula and database ID and structure of the compound respectively. Hexane extracted 5 compounds from Au mine tailings. These compounds contain various functional groups. N-(2-ethenylsulfonylethyl)ethane-1,2-diamine contains a sulfonyl and amine functional groups. N-methoxyundecan-2-imine contains an imine functional group. N-(2-Chloro-6-fluorobenzyl)-2-methyl-1-propanaminium contains a benzyl ring with two alkyl halides (Chlorine and Fluorine) flanking the benzyl ring, and an amine functional group.

Eupatoriochromene has insecticidal activity, the compound also retards seed germination, reduces weed and plant seedling growth (Merrill, 1989). Eupatoriochromene extracted from medicinal plants in Ghana has recently shown to be an bioactive compound in antitrypanosomal treatment as indicated by the work done by Twumasi et al., (2020). Eupatoriochromene is a chromene class of compounds as a secondary metabolite chromenes contain biological and pharmacological properties demonstrating antimicrobial, anti-inflammatory, antioxidant and cytotoxic activities (Sánchez-Ramos et al., 2021). Pro-Arg compound is an amino acid chain consisting of amino acids proline and arginine.

Non-volatile organic compounds were extracted from Au mine tailings using DCM, ethyl acetate and hexane as extraction solvents. A total of 9 organic compounds were identified via LC-QTOF-MS, with four compounds extracted by DCM, one compound extracted by ethyl acetate and five compounds extracted by hexane. The results indicate that hexane and DCM has a higher affinity for organic compounds as compared to ethyl acetate during the extraction. These compounds have various functional groups due to their complexity in molecular formula and structure, A few dominant functional groups present in the 9 identified compounds are; benzene rings, amides (RCONR2), sulfonyl (RSO2R), amines (RNH2), imines [RC(=NH)R'], Tautomycin a PP1 inhibitor was also identified and Eupatoriochromene a bioactive secondary metabolite and insecticide was identified.

4.2.3.2 Organic compounds identified by GC/MS

Screening of volatile organic compounds extracted from Au mine tailings for the purpose of identifying organic compounds of environmental concern. Solid-liquid extraction was performed using three (3) organic solvents such as dichloromethane (DCM), ethyl acetate and hexane to validate the extraction and recover substantial organic compounds. Separation of volatile compounds was achieved using gas chromatography (GC) and identification occurred through mass spectrometer (MS). GC-MS is commonly used for determination of volatile analytes in environmental samples (Pitarch et al., 2016).

Volatile Compounds detected via GC/MS which were extracted by DCM (figure 4.5), ethyl acetate (figure 4.6) and hexane (figure 4.7) are depicted with their RT, name, molecular formula and structure. A total of 18 organic compounds were identified, of which 6 compounds were extracted using DCM, 5 compounds were extracted using ethyl acetate and 7 compounds were extracted using hexane. The hexane extraction yielded the most volatile organic compound whereas the least compounds were extracted using ethyl acetate.

Volatile organic compounds were analysed on the gas chromatography time of flight mass spectrometer. The above mentioned samples prepared for GC were analysed for non-volatile compounds.

Au mine tailing samples were analysed for organic compounds present in the samples. Solid-liquid extraction was used to extract organic compounds from the samples with the use of organic solvents such as hexane, dichloromethane (DCM) and ethyl acetate. Samples were analysed and compounds were identified via GC-TOF-MS. Tables below indicate analysed results of compounds identified using GC-TOF-MS.

Table 4. 5: Compounds of concern to environmental contamination extracted using DCM.

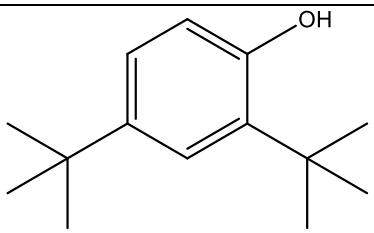
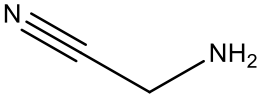
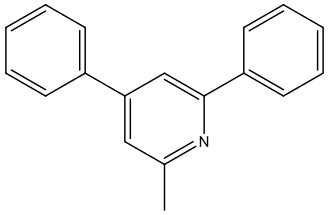
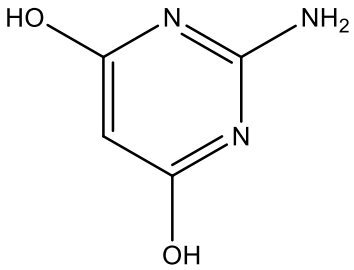
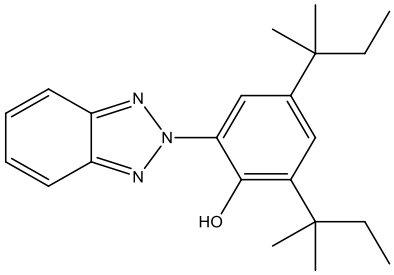
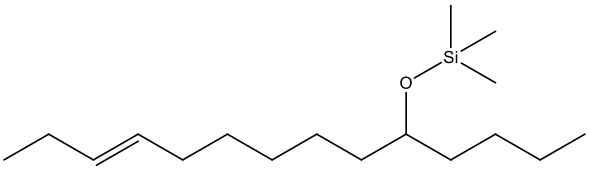
RT (min)	Compound Name	Formula	Structure
12:42.00	2,4-Di-tert-butylphenol	C14H22O	
17:36.00	Aminoacetonitrile	C2H4N2	
22:57.00	Pyridine, 2-methyl-4,6-diphenyl-	C18H15N	
23:30.00	2-Amino-4,6-dihydroxypyrimidine	C4H5N3O2	
25:48.00	Phenol, 2-(2H-benzotriazol-2-yl)-4,6-bis(1,1-dimethylpropyl)-	C22H29N3O	
27:03.00	E-11-Tetradecenol, trimethylsilyl ether	C17H36OSi	

Table 4.5 indicates volatile organic compounds extracted using DCM and identified via GC/MS, for compounds that contained functional groups with environmental concerns. DCM enabled the extraction of compounds with functional groups with environmental concern such as phenols (2,4-Di-tert-butylphenol; Phenol, 2-(2H-benzotriazol-2-yl)-4,6-

bis(1,1-dimethylpropyl)-), nitriles (aminoacetonitrile); pyridine (pyridine, 2-methyl-4,6-diphenyl-), pyrimidine (2-amino-4,6-dihydroxypyrimidine) and ether (E-11-Tetradecenol, trimethylsilyl ether).

Table 4. 6: Compounds of concern to environmental contamination extracted using ethyl acetate.

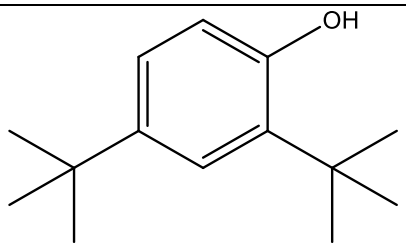
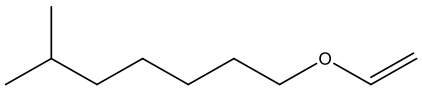
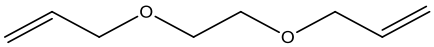
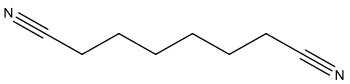
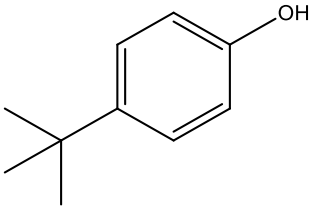
RT (min)	Compound name	Molecular formula	Structure
12:42.00	2,4-Di-tert-butylphenol	C14H22O	
13:30.00	Ether, 6-methylheptyl vinyl	C10H20O	
14:48.00	Ethylene glycol diallyl ether	C8H14O2	
20:36.00	Octanedinitrile	C8H12N2	
30:03.00	4-tert-Butylphenol, TMS derivative	C13H22OSi	

Table 4.6 indicates volatile organic compounds extracted using ethyl acetate and identified via GC/MS, for compounds that contained functional groups with environmental concerns. Ethyl acetate enabled the extraction of compounds with functional groups of environmental concern such as phenols (2,4-di-tert-butylphenol and 4-tert-butylphenol), nitrile (octanedinitrile) and ethers (ethylene glycol diallyl ether and ether, 6-methylheptyl vinyl).

Table 4. 7: Compounds of concern to environmental contamination extracted using hexane.

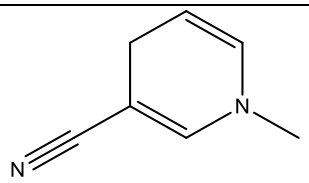
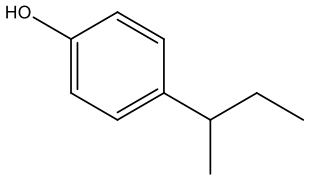
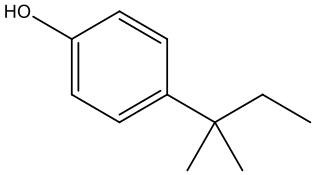
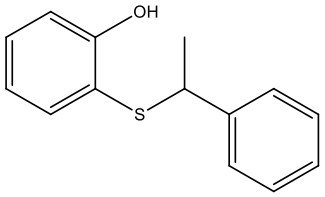
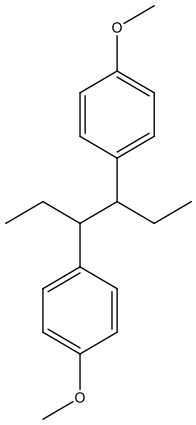
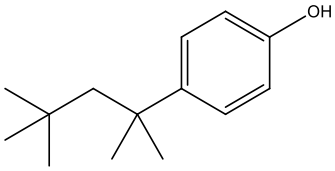
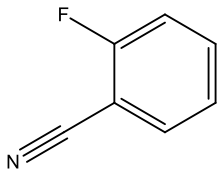
RT (min)	Compound Name	Molecular formula	Structure
12:24.00	3-Pyridinecarbonitrile, 1,4-dihydro-1-methyl-	C7H8N2	
14:48.00	Phenol, 4-(1-methylpropyl)-	C10H14O	
15:03.00	Phenol, 4-(1,1-dimethylpropyl)-	C11H16O	
15:06.00	Phenol, 2-[(1-phenylethyl)thio]-	C14H14OS	
15:24.00	Hexestrol dimethyl ether	C20H26O2	
16:03.00	Phenol, 4-(1,1,3,3-tetramethylbutyl)-	C14H22O	
32.934	Benzonitrile, 2-fluoro-	C7H4FN	

Table 4.7 indicates volatile organic compounds extracted using hexane and identified via GC/MS, for compounds that contained functional groups of environmental concerns. haxane enabled the extraction of compounds with functional groups with environmental concern such as phenols (phenol, 4-(1-methylpropyl)-; phenol, 4-(1,1-dimethylpropyl)-; Phenol, 2-[(1-phenylethyl)thio]- and phenol, 4-(1,1,3,3-tetramethylbutyl)-), 3-Pyridinecarbonitrile, 1,4-dihydro-1-methyl- contains a pyridine and a nitrile functional group, the other compound containing a nitrile group is benzonitrile, 2-fluoro; and hexestrol dimethyl ether contained an ether functional group.

Environmental contamination is a result of various industries and anthropological activities that dispose of waste into the environment with little or no treatment. Organic compounds were extracted from Au mine tailings and identified as shown in the tables above using DCM, ethyl acetate and hexane as extractants. The tables above show organic compounds that belong to the functional groups of ether; pyridines; pyrimindines; phenols and; nitriles which are of an environmental concern

A total of 18 volatile organic compounds with functional groups of environmental concerns were identified, of which 6 were extracted using DCM, 5 were extracted using ethyl acetate and 7 were extracted using hexane as extraction solvents.

The agrochemical and pharmaceutical industries utilize pyridine as an intermediate or raw material for the production/synthesis of vitamins, sulphonamides, disinfectants, explosives, dyes, rubber and paint (Xue et al., 2020). Wastewater discharge from the above production and industries leads to pyridine being disposed into the environment (Wang et al., 2018). The United States Environmental Protection Agency has listed and classified pyridine as a priority pollutant due to its toxic, teratogenic and carcinogenic properties (Wang et al., 2018; Xue et al., 2020)

Pyrimidine is another ingredient that is used in the synthesis of vitamins (vitamin B1). Pyrimidine present in pharmaceutical waste are bioactive, polar and persistent which pose an enormous threat to all life forms and the environment (Wang et al., 2019). Phenol is used in the production of petrochemicals, pharmaceuticals, herbicides, dyes, cosmetics and, pesticides as well as household products (Zango et al., 2020).

Petroleum refinery wastewater contains phenols (Salcedo et al., 2019), the use and disposal of products containing phenols also contributes to the presence of phenols in the environment (Nthunya et al., 2019). Phenolic discharge in the environment is a concern for human health, with toxic effects on humans being; skin and eye irritation, respiratory complications, weight loss, diarrhoea, vertigo and, dark coloration of urine (Zango et al., 2020). The US Environmental Protect Agency and the European Community included some phenols on their priority pollutants lists due to their high toxicity, persistence in the environment and potential carcinogenicity (Salcedo et al., 2019).

Wastewaters from urban and agricultural activities, metals manipulation and mining activities contains nitriles. Nitriles are also found in wide ranging industrial products such as; solvents, plastics, synthetic rubber, herbicides and pharmaceuticals (Heidari and Asoodeh, 2019). Nitriles can produce carcinogenic changes in humans by acting on cellular DNA (Heidari and Asoodeh, 2019). Benzonitrile is a synthetic nitrile which has a vast application in the industrial and manufacturing industries as solvents and intermediates for the production of pharmaceuticals, dyes, rubber, agrochemicals, perfumes, textiles, resins, melamine, jet fuel additive, nickel platting and herbicides. A vast network of industries utilizing benzonitriles for their productions leading to discharged wastewater contaminating the environment and directly threatens all life forms, thus making it a health hazard due to their high toxicity, mutagenic and carcinogenic attributes (Mukram et al., 2015).

4.2.4 Summary

Extraction of organic molecules from Au mine tailings was done via a solid-liquid extraction, with hexane; DCM and ethyl acetate. Identification of the extracted compounds was done by LC-QTOF-MS for non-volatile and GC-TOF-MS for volatile organic compounds. A total of 27 organic compounds were extracted and identified. LC-QTOF-MS identified 9 non-volatile organic compounds, while GC-QTOF-MS identified 18 volatile organic compounds with functional groups of environmental concerns. Compound classes with environmental significance were regarded as important, these classes are: ethers; pyridines; phenols and nitriles. Benzonitrile was selected as the compound of interest for further studies.

4.3 Preliminary biodegradation trials on aminobenzonitrile

4.3.1 Introduction

Preliminary biodegradation trials were conducted on aminobenzonitrile. These trials were done to identify which bacterial isolates possess the ability to degrade aminobenzonitrile in a setting where nitrogen and carbon sources were absent.

4.3.2 Objectives

1. Identify which bacterial species isolated from Au mine tailings are able to degrade aminobenzonitrile.

4.3.3 Results and discussion

The identified bacterial species were subjected to preliminary biodegradation trials, with aminobenzonitrile as the compound of interest. The preliminary trials were conducted by inoculating 10 % (v/v) of previously grown bacterial cultures into M.M, which contained 100 mg/L aminobenzonitrile and incubated at 30 °C with agitation at 180 rpm for 72 hrs. Thereafter, the concentration of ammonium was monitored during the course of the biodegradation process. The ammonium concentration was analysed since the degradation of aminobenzonitrile ultimately results mainly in the formation of ammonia. This was done to determine the organisms which were able to degrade aminobenzonitrile.

Table 4. 8: Ammonium formation by bacterial species after preliminary biodegradation trials with aminobenzonitrile.

Sequence ID	Bacterial species	Ammonium formed (mg/L)
BRB M1 (MW300681)	*P. shunpengii*	49.7
BRB M2 (MW300682)	*B. pumilus*	76.1
BRB M3 (MW300683)	*B. safensis*	>75 (79.9)
BRB M4 (MW300684)	*B. thuringiesis*	66.5
BRB M5 (MW300685)	*B. cereus*	74.5

Nitrile degradation results in the formation of carboxylic acid and ammonia as products. The preliminary biodegradation trials detected the ammonium formed in the M.M as a

result of aminobenzonitrile degraded by the bacterial species. Formation of ammonium indicates the bacterial species' ability to metabolize aminobenzonitrile, thus indicating the bacterial species ability to utilize aminobenzonitrile as a carbon/nitrogen source (Mukram et al., 2015). The bacterial species indicated in table 3 namely: *P. shunpengii*; *B. pumilus*; *B. safensis*; *B. thuringiesis*; and *B. cereus* were able to metabolize aminobenzonitrile. Kao et al., (2006), demonstrated that *Klebsiella oxytoca* strain was unable to biodegrade benzonitrile as no ammonia production was observed which indicated no enzymatic activity while Egelkamp et al., (2019) observed that *Paenibacillus* was able to grow in 4-hyoxybenzonitrile supplemented cultures. Benedik and Sewell, (2018) predicted *Paenibacillus* contains nitrilase-encoding genes with close homology to cyanide dihydratase. *Bacillus pumilus* C1 (AF492815) contains cyanide dihydratase and the protein sequence homologs were seen in *Bacillus safensis* and *Bacillus thurengiesis* (Benedik & Sewell, 2018); this explains the ability of *P. shunpengii; B. pumilus; B. safensis* and; *B. thuringeiesis* to breakdown benzonitrile. *B. safensis* yielded the most ammonium with 79.9 mg/L, and *Paenibacillus shunpengii* yielded the least ammonium with 49.7 mg/L. Based on the preliminary trial results, all the tested bacterial species were able to biodegrade aminobenzonitrile, thus further biodegradation tests proceeded with all the bacterial species pooled together to form a consortium.

4.3.4 Summary

The biological degradation of nitriles results in carboxylic acid and ammonia being formed as products. Thus the preliminary biodegradation trials tested for ammonium formation within the M.M as an indicator of the bacterial species ability to degrade aminobenzonitrile. All the bacterial species namely; P. shunpengii, B. pumilus, B. safensis, B. thuringiesis, and B. cereus, isolated from Au mine tailings produced ammonium from aminobenzonitrile as a nitrogen and carbon source. B. safensis yielded the most ammonium through degradation of aminobenzonitrile.

4.4 Optimisation of aminobenzonitrile biodegradation process

4.4.1 Introduction

Optimisation of the degradation process of aminobenzonitrile by the bacterial consortium established enables for the bioprocess to be conducted in the most efficient and productive manner. The influence of independent factors/variables are assessed and optimised which enables the bioprocess to function at its best. Design Expert software enables for optimisation of variables through response surface methodoly (RSM) using central composite design (CCD).

The variables under investigation were temperature, pH and substrate concentration (aminobenzonitrile). These factors were subjected to optimisation studies for maximum performance by the bioprocess (bacterial consortium).

4.4.2 Objectives

1. Asses the biodegradation of aminobenzonitrile by bacterial consortium isolated from Au mine tailings.
2. Optimise independent factors/variables (temperature, pH and substrate concentration) using response surface methodology.

4.4.3 Results and discussion

4.4.3.1 Response surface methodology (RSM)

The central composite design (CCD) of RSM was used to study the interactions between the factors/variables and thereby determining their optimal conditions after 72 hrs. The factors under investigation were; temperature, pH and substrate concentration. The CCD constructed a 20-run experiment (table 3.2) using the above factors and their limits (Table 3.1). The BRE % of aminobenzonitrile by the bacterial consortium was obtained as per instructions stated in section 3.5.4.2 (Quantification of aminobenzonitrile using GC/MS), a standard curve was constructed and the concentrations before and after biodegradation test experiments were extrapolated (Appendices). Table 4.9 shows the actual data obtained from the experiment of the effects of temperature, pH and substrate concentration on the biodegradation of aminobenzonitrile. The experimental results were analysed through RSM in order to obtain the best response. The experimental results showed a biological removal efficiency of 91 % under the conditions of run 1, after a 72

hour incubation, which suggests that medium substrate concentration (100 mg/L aminobenzonitrile) and pH (8.5) at low temperature (21.59 °C) was most effective in biodegradation of aminobenzonitrile. Table 4.13 indicates the actual and predicted results.

Table 4. 9: Central composite design (CCD) for the three factors and response result (BRE).

Standard order	Run	Temperature C	pH	Concentration mg/L	BRE (%)
9	1	21.59	8.50	100.00	91
4	2	35.00	10.00	50.00	82
18	3	30.00	8.50	100.00	13
2	4	35.00	7.00	50.00	61
13	5	30.00	8.50	15.91	55
17	6	30.00	8.50	100.00	48
5	7	25.00	7.00	150.00	38
1	8	25.00	7.00	50.00	0
12	9	30.00	11.02	100.00	33
20	10	30.00	8.50	100.00	47
14	11	30.00	8.50	184.09	14
16	12	30.00	8.50	100.00	91
15	13	30.00	8.50	100.00	52
19	14	30.00	8.50	100.00	68
11	15	30.00	5.98	100.00	49
7	16	25.00	10.00	150.00	23
3	17	25.00	10.00	50.00	0
8	18	35.00	10.00	150.00	51
6	19	35.00	7.00	150.00	48
10	20	38.41	8.50	100.00	36

Table 4. 10: Central composite design used for each factor.

Factor	Name	Units	Low Actual	High Actual	Low Coded	High Coded	Mean	Std. Dev.
A	Temperature	°C	25.00	35.00	-1.000	1.000	30.000	4.132
B	pH		7.00	10.00	-1.000	1.000	8.500	1.240
C	Concentration	mg/L	50.00	150.00	-1.000	1.000	100.000	41.317

Response	Name	Units	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio
Y1	BRE	%	Polynomial	0.000	91.000	45.000	25.886	N/A

4.4.3.2 Statistical analysis

Statistical analysis was done using analysis of variance (ANOVA) which evaluated the model to assess each independent factor as input and the biodegradation of aminobenzonitrile by the bacterial consortium as output/result. A quadratic model for analysis was used and an equation was obtained that could relate aminobenzonitrile biodegradation as a measured output to the variables as input.

$$Y = 53.43 + 6.48A – 1.31B – 3.80C + 4.88AB – 13.12AC – 4.12BC + 1.96A2 – 6B2 – 8.3C2 \quad (4.1)$$

The model was assessed using ANOVA shown in table 4.11. The Model F value of 0.48 and p value of 0.8586 indicates that the model used was not significant. A p value less than 0.05 indicates significance. There are no significant terms in this model. These analyses indicate that there is no direct correlation between the input variables (temperature, pH and substrate concentration) and the result in terms of amionobenzonitrile biodegradation as an output/result. The results indicates that the biodegradation of aminobenzonitrile by the bacterial consortium occurs independently from the factors, temperature, pH and substrate concentration, under study. The lack of fit F value of 1.81 implies the lack of fit is not significant to the pure error and the model fits.

Table 4.12 indicates the model has a determination coefficient (R2) of 0.3011 which indicates that 30.11% of variability in the response is attributed to independent variables. The low adj determination coefficient (Adj R2 = -0.3279) also suggests the model is not significant (Agarwal and Nigam, 2017). The predicted determination coefficient (Pred R2 = -2.7782) implies that the overall mean is a better predictor of the response than the current method. The coefficient of variance (CV) obtained a very high CV value of 68.01 with implies low confidence of the experiments. The high CV indicates that there was a great dispersion around the mean. The high variability shown by the CV could be due to the lack of detection of treatment effects.

Table 4. 11: ANOVA table for quadratic model.

Source	Sum of squares	df	Mean square	F value	p-value Prob > F
Model	4035.67	9	448.41	0.48	0.8586
A-Temperature	573.52	1	573.52	0.61	0.4520
B-pH	23.48	1	23.48	0.025	0.8773
C-Concentration	197.64	1	197.64	0.21	0.6558
AB	190.13	1	190.13	0.20	0.6619
AC	1378.12	1	1378.12	1.47	0.2530
BC	136.13	1	136.13	0.15	0.7110
A^2	55.13	1	55.13	0.059	0.8132
B^2	518.63	1	518.63	0.55	0.4739
C^2	992.10	1	992.10	1.06	0.3276
Residual	9366.33	10	936.63		
Lack of Fit	6035.50	5	1207.10	1.81	0.2650
Pure Error	3330.83	5	666.17		
Cor Total	13402.00	19			

Table 4. 12: Statistical analysis terms for the model.

Std. Dev.	30.60	R2	0.3011
Mean	45.00	Adj R2	-0.3279
C.V. %	68.01	Pred R2	-2.7782
PRESS	50634.91	Adeq Precision	2.262

Normality of residuals was checked by normal probability plot of studentized residuals and the studentized residuals versus predicted values were checked using model diagnostic tools within the Design Expert software. The normal probability plot of residuals is used to judge model adequacy. As shown in Figure 4.5 the residual plot approached along a straight line indicating normality was satisfactory for this model.

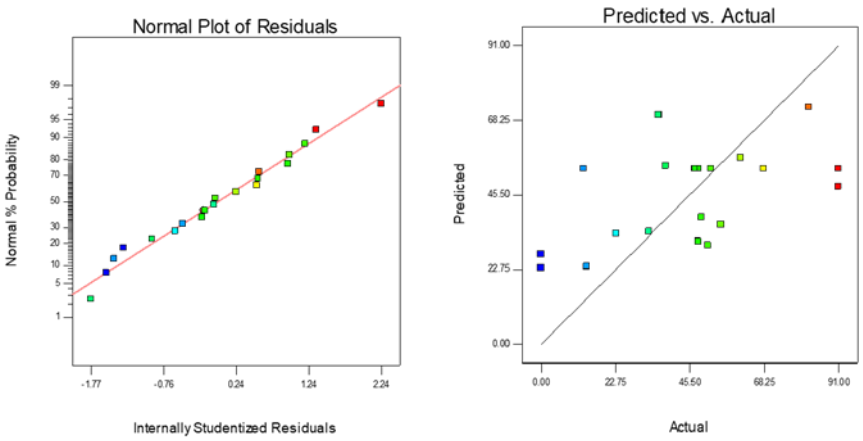


Figure 4. 5: Graph comparing the normality % probability and internal studentized residuals (A) and the predicted response with the actual response (B).

Table 4. 13: Actual and predicted responses obtained using CCD.

Standard Order (Std.)	Actual value	Predicted value
1	0.000	27.35
2	61.00	56.81
3	0.000	23.22
4	82.00	72.18
5	38.00	54.24
6	48.00	31.20
7	23.00	33.62
8	51.00	30.08
9	91.00	48.06
10	36.00	69.86
11	49.00	38.66
12	33.00	34.25
13	55.00	36.36
14	14.00	23.56
15	52.00	53.43
16	91.00	53.43
17	48.00	53.43
18	13.00	53.43
19	68.00	53.43
20	47.00	53.43

4.4.3.3 Graphical representation of the response surface model

Response surface curves/graphs were plotted to determine the interaction effects of the variables. Figure 4.6 illustrates the 3D graphs of the interactions between aminobenzonitrile biodegradation (BRE) and the variables studied which are, temperature, pH and substrate concentration. The 3D response surface and corresponding contour plots in Figure 4.6a and b illustrates the interaction between varying pH and temperature, whereby with an increase in temperature and pH there is an increase in the biodegradation of aminobenzonitrile. Figure 4.6c and d depicts the 3D response surface and corresponding contour plots for the interaction between temperature and

concentration, which implies there is no direct influence between temperature and concentration on the outcome of biodegradation of aminobenzonitrile, as a higher BRE is obtained at a higher temperature with a lower substrate concentration. Figure 4.6e and f depicts the interactions between pH and substrate concentration shown in their 3D response surface and contour plots, which depicts there is no direct influence between the two variable (pH and substrate concentration).

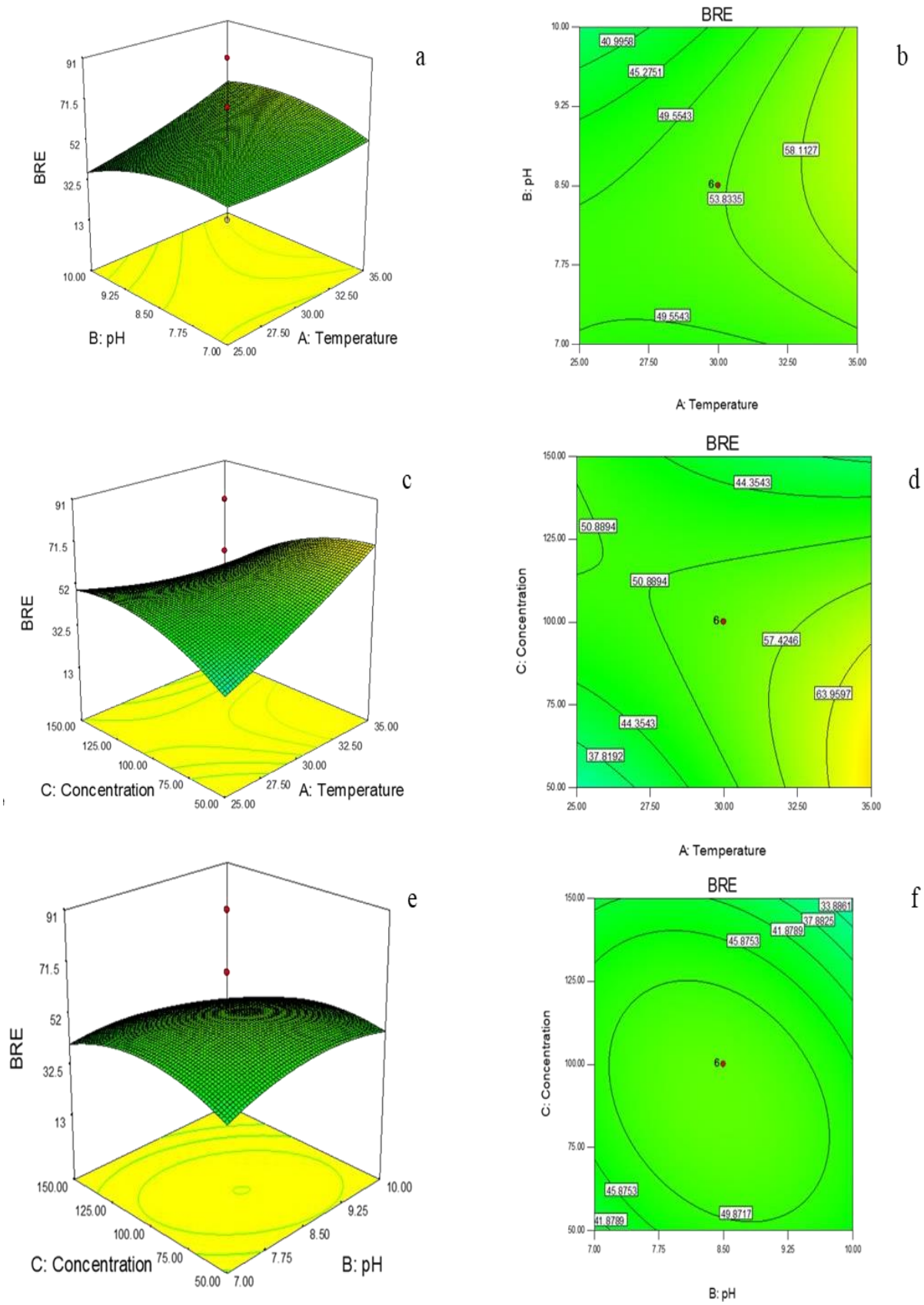


Figure 4. 6: 3-D plots a, c, e and contour plots b, d, f illustrating the interaction between the independent variables on aminobenzonitrile biodegradation (BRE).

4.4.3.4 Process optimisation

Design expert software was used to optimise the bioprocessing of aminobenzonitrile, by assessing the input variables to obtain a response to maximise aminobenzonitrile biodegradation. The optimum operation conditions for the three variables were obtained by the numerical optimisation (Agarwal and Nigam, 2017) and are presented by the ramps diagram below, in figure 4.7.

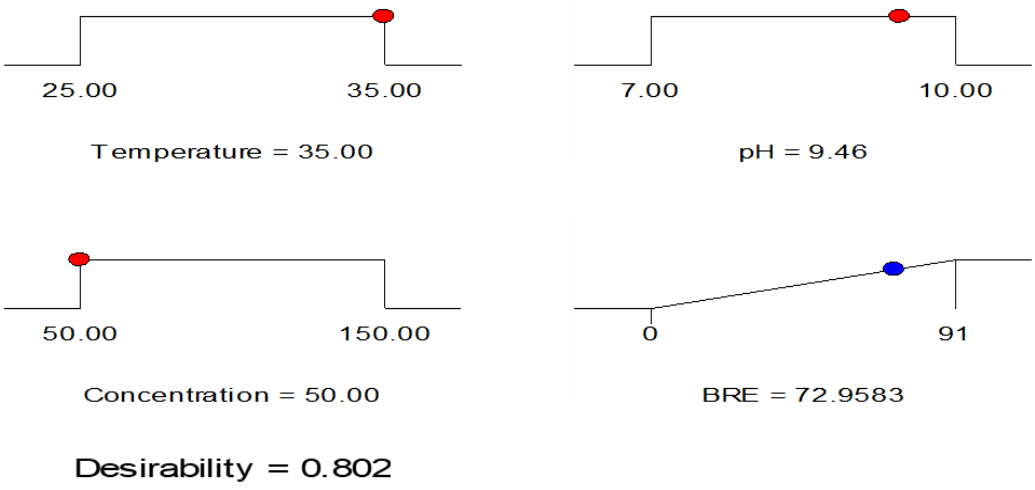


Figure 4. 7: Numerical optimisation of the input variables: temperature, pH and substrate concentration.

The optimal conditions for temperature, pH and substrate concentration (aminobenzonitrile) for the biodegradation of aminobenzontirle by the bacterial consortium over 72 hrs. determined by numerical optimisation were 35 °C, 9.46 and 50 mg/L respectively with the maximum biological removal efficiency (BRE %) of 73.

Li et al., (2007), examined the nitrile degrading capabilities of a microbial sludge consortium, their results indicated an initial lag phase for 40 h followed by a gradual decrease in benzonitrile from 2g/L to 157.2 mg/L at 314 h, these experiments were conducted under conditions of 25 °C and pH 7. Rhodococcus sp. MTB5 was able to be grown in a mineral salt media with benzonitrile as the sole source of carbon and nitrogen, the complete utilization of 30 mM benzonitrile was observed within 42 h with products of ammonia at the maximum of 4 mM stated by Mukram et al., (2015). Benzonitrile

metobilsm studies through Klebsiella pneumonia was done by Nawaz et al., (1992) indicated K. pneumonia metabolised 8.4 mM benzonitrile to 4.0 mM benzoic acid and 2.7 mM ammonia. Veselá et al., (2010), demonstrated the ability of soil actinobacteria *Rhodococcus rhodochrous* PA-34; *Rhodococcus* sp. NDB 1165 and *Nocardia globerula* NHB-2 activity towards benzonitrile, whereby resting cell suspensions of the mentioned strains were able to eliminate/metabolise benzonitrile and benzonitrile analogues chloroxynil (3,5-dichloro-4hydroxybenzonitrile), bromoxynil (3,5-dibromo-4-hyrdoxybenzonitrile) and ioxynil (3,5-diiodo-4-hydroxybenzonitrile) at 30 °C and pH 8.0 yeilding products of their corresponding bezoic acids. Sulistinah and Sunarko, (2020), demonstrated that *Rhodococcus pyridionvorans* strain I-Benzo isolated from leather tanning waste is able to degrade benzonitrile at pH 7.2 at room temperature over 180 min. *Nocardia* sp. 108 was able to hydrate p-aminobenzonitrile with the optimal temperature and pH being 28 °C and pH 7 (Wang et al., 2007) with p-aminobenzonitrile being exhibiting 8.98% hydration after 5 min.

The bacterial consortium used in this study have shown they contain the ability to biodegrade aminobenzontirile. Over the course of the 20-run experiments seen in table 4.3 it is observed that aminobenzonitrile is biodegraded by the consortium under various conditions. The lowest achieved BRE % by the consortium was 13 % under conditions, 30 °C, pH 8.5 and 100 mg/L substrate concentration (aminobenzonitrile). Conditions which we observed inactivity by the consortium were: 25 °C, pH 7 and 50 mg/L ; as well as 25 °C, pH 10 and 50 mg/L both runs produced 0 % BRE. The highest observed BRE % was 91 % under the conditions of, 21.59 °C, pH 8.5 and 100 mg/L substrate concentration. It is observed that the bacterial consortium contains biodegrading ability for aminobenzonitrile under a wide range of pH and temperatures ranging from 5.98 to 11 and 21 to 38 °C.

4.4.4 Summary

The influence of independent variables such as temperature, pH and substrate concentration on the biodegradation of aminobenzonitrile were tested with the aim of optimising process efficiency. Response surface methodology was used to obtain the optimum conditions for temperature, pH and substrate concentration (aminobenzonitrile) were found to be 35 °C, 9.46 and 50 mg/L respectively, with a maximum biological

removal efficiency (BRE) of 73 % over a 72 hour period. A quadratic model was obtained from Design Expert 7.0. Statistical analysis via ANOVA indicated the variables do not have a direct relation to the outcome result.

4.5 Biodegradation products to assist in pathway determination of aminobenzonitrile biodegradation by the bacterial consortium.

4.5.1 Introduction

Biodegradation products are formed as a result of microorganisms' ability to degrade/metabolise a substrate. The assessment of these biodegradation products contributes insights into the possible enzymatic pathways utilised by the organisms under study. Biodegradation products were identified through an analytical method GC- MS analysis and assessed as a basis for possible enzymatic pathway determination.

For the purpose of this study (biodegradation of aminobenzonitrile by a consortium of bacterial species isolated from Au mine tailings) we further investigate the possible pathway of aminobenzonitrile biodegradation by comparing the GC-MS chromatogram results obtained from the initial and the final sample.

4.5.2 Objectives

1. Identify biodegradation products of aminobenzonitrile degradation, and possible enzymatic pathway utilised by the bacterial consortium based on the biodegradation products.

4.5.3 Results and discussion

Biodegradation products of aminobenzonitrile, by the bacterial consortium, was determined using GC/MS. Separation of compounds was done using GC equipped with DB WAX UI column and ran on conditions mentioned in the Materials and Methods section 3.5.3.1. Identification of compounds was done by MS, as discussed in section 3.5.3.2. The following chromatograms illustrate compound separation obtained by GC for initial (prior to biodegradation test) and final (after 72 hrs.) sample (figure 4.8a and 4.8b).

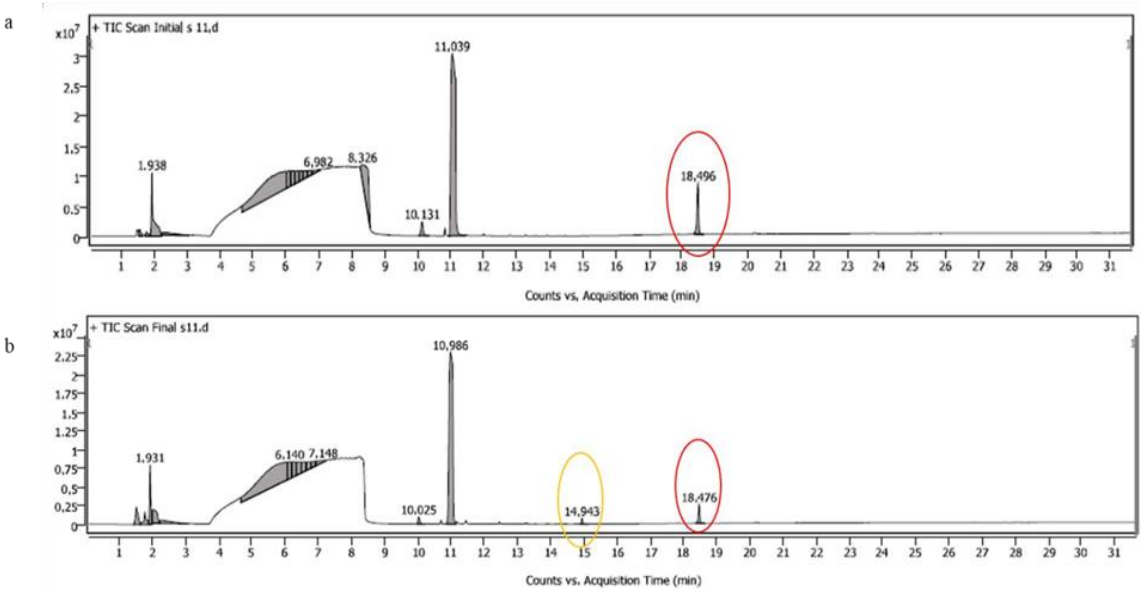


Figure 4. 8: GC chromatogram illustrating compound separation conducted from the biodegradation test of aminobenzonitrile. (a) Indicates compounds present in M.M containing aminobenzonitrile sample prior to the addition of bacterial consortium. (b) Indicates compounds present after 72 hrs. of incubation with 10 % (v/v) of bacterial consortium.

A comparison of chromatograms 4.8a and 4.8b can be used to depict changes in the media after 72 hrs. incubation with the bacterial consortium. Various peaks (compounds) are found in Figure 4.8a, eluted out with RT from 1 min up to 19 min, with notable peak at 18.4 min representing and identified as aminobenzonitrile (via MS) the compound of interest. The chromatogram 4.8b has a similar elution profile as to 4.8a with two notable changes. The first difference we see is a peak with the RT of 14.9 min, based on the MS data this peak identifies as 2-piperidinone with an *m/z* of 99.03. The second difference is the intensity of the aminobenzonitrile at RT of 18.4 min being reduced in comparison to 4.8a, this indicates that aminobenzonitrile was catabolized by the bacterial consortium.

Table 4. 14: Summary of GC-MS data from the chromatograms in figure 4.8a and 4.8b.

Figure 4.8a

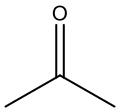
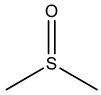
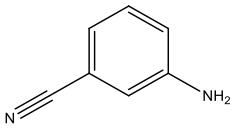
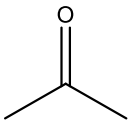
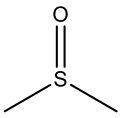
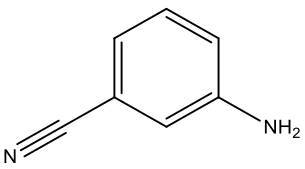
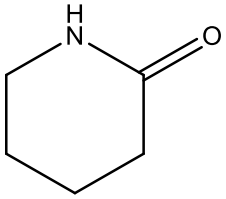
Peak	RT (min)	Compound	Molecular formula	*m/z*	Structure
1	1.938	Acetone	C_3H_6O	58.03	
2	11.039	Dimethyl sulfoxide (DMSO)	C_2H_6OS	80.0398	
3	18.496	Benzonitrile,m-amino	$C_7H_6N_2$	118.0399	

Figure 4.8b

Peak	RT (min)	Compound	Molecular formula	*m/z*	Structure
1	1.931	Acetone	C_3H_6O	58.03	
2	10.986	Dimethyl sulfoxide (DMSO)	C_2H_6OS	80.0398	
3	18.476	Benzonitrile,m-amino	$C_7H_6N_2$	118.0399	
5	14.943	2-Piperidinone	C_5H_9NO	99.0399	

Biodegradation test of aminobenzonitrile by the said bacterial consortium was analysed by GC-MS for compound separation and identification of biodegradation products, to assist possible enzymatic pathway determination. The chromatogram in figure 4.8a illustrates distinct peaks corresponding to compounds within the M.M before the addition of the bacterial consortium. With the retention times (RT) at 1.938 min; 10.966 min and 18.45 min. Based on the MS data, a peak with RT of 1.938 min. identifies as acetone with *m/z* 58.081; peak with RT of 11.039 min. as dimethyl sulfoxide (DMSO) with *m/z* 80.0398 and peak with RT of 18.496 min. as benzonitrile,m-amino wit *m/z* 118.039. Figure 4.8b illustrates the peaks found in the chromatogram after 72 h of incubation with the bacterial consortium. Notably one more distinct peak are found in figure 4.8b, based on the GC-MS for figure 5b, 14.923 min, the MS data identified this peak as 2-piperidinone with *m/z* 99.0399 (Table 4.14).

From the initial chromatogram (figure 4.8a) table 4.14 indicates that 3 major compounds were identified as Acetone, DMSO and; benzonitrile, m-amino. These compounds are in-line with contents present in the initial tests (before the addition of bacterial consortium). Acetone identified was due to the injection solvent used for GC, to inject the sample for analysis. Aminobenzonitrile is insoluble in water thus a solvent capable of dissolving aminobenzonitrile while also being able to be soluble in water was required to conduct the experiments thus DMSO was used as a solvent in which aminobenzonitrile is soluble in and soluble in water. The last compound found in the initial sample was benzonitrile,m-amino, which is the compound of interest for biodegradation study. The above 3 compounds were also found in final test chromatogram (figure 4.8b) with the addition of one compound (Table 4.14). The compound was identified as 2-Piperidinone respectively. Based on the results obtained we see that acetone, DMSO and benzonitrile,m-amino are found in both chromatograms and the final chromatogram contained 2-Piperidinone as addition, therefore it can be deduced that 2-Piperidinone was formed as a biodegradation product of benzonitrile,m-amino after the 72 hrs. incubation with bacterial consortium (figure 4.8b).

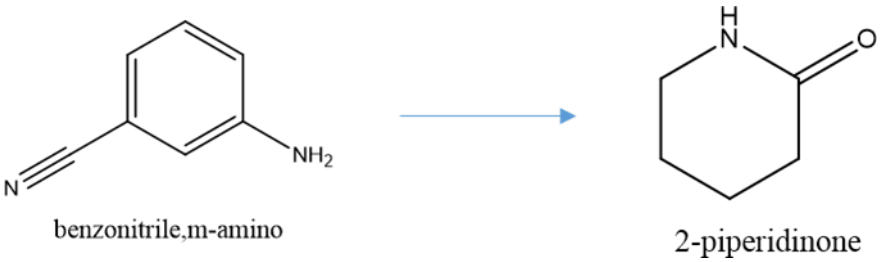


Figure 4. 9: Benzonitrile,m-amino degraded to 2-piperidinone by the bacterial consortium.

Microbial degradation of nitriles follows two enzymatic pathways. One pathway is a bi-enzyme pathway utilizing nitrile hydratase (NHase) which catalyses nitrile to an amide, which acts as a substrate for amidase (second enzyme), which is hydrolysed to carboxylic acid and ammonia. In second pathway, nitriles are directly hydrolysed to carboxylic acid and ammonia by nitrilase (Mukram et al., 2015). Based on the results obtained from GC/MS chromatogram and the standard curve, it was observed that experiments that contained a decrease in benzonitrile,m-amino yielded a product/compound 2-piperidinone. 2-piperidinone (2-piperidone, δ-valerolactam) is cyclic amide which is classified further as a delta-lactam (Al-Bahadily et al., 2019; Lister and Owen, 1973). Benzonititrile degradation by *Rhodococcus* sp. MTB5 strain follows the bienzymatic pathway utilizing NHase/amidase yielding benzamide and benzoinic acid (Mukram et al., 2015). Nawaz et al., (1992) reported that *Klebsiella pneumonia* strain was able to catabolise benzonitrile to benzoic acid via the NHase/amidase pathway, there was no detection of benzamide but the cell extracts contained NHase and amidase activity which suggests benzamide is an intermediate of benzonitrile metabolism. The work of Sulistinah and Riffiani, (2018) indicated the conversion of benzonitrile to benzamide then to benzoic acid involving NHase and amidase by *Rhodococcus pyridinivoras* strain SB1D1.

4.5.4 Summary

The bacterial consortium used in this study biodegrades aminobenzonitrile to form 2-piperidinone. The biodegradation product, 2-piperidinone is a cyclic amide, indicates that an amide is formed from the biodegradation of aminobenzonitrile by the bacterial consortium. Formation of an amide (2-piperidinone, cyclic amide) indicates that the bacterial consortium follows the enzymatic pathway which degrades nitriles to an amide by NHase.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Nitriles are known environmental contaminants, with benzonitrile being one of the most widespread synthetic nitrile used in the production of various products. The vast application of benzonitrile in products leads to environmental contamination via the wastewater discharge into the environment with little pre-treatment. The hypothesis of study, was that microorganisms isolated from Au mine tailings are able to degrade organic compounds (nitriles). The study approach included identifying microorganisms and organic compounds in Au mine tailing habitat, preliminary biodegradation trials against benzonitrile (as the organic compound of interest), optimization of physicochemical parameters for biodegradation of benzonitrile and possible pathway determination of benzonitrile degradation by microorganisms. The data shows that five microorganisms were successfully identified namely, *B. pumilus, B.safensis, B.thuringiesis, B. cereus* and *P. shungpengii*. A total of 27 organic compounds were identified, from Au mine tailings, with compounds belonging to classes, ethers, pyridines, pyrimidines, phenols and nitriles which are of environmental concern. Preliminary biodegradation trials against aminobenzonitrile as the organic compound of interest showed all five bacterial species are able to degrade aminobenzonitrile yielding ammonium as a product. The optimal biodegradation conditions for temperature, pH and substrate concentration were 35 °C, 9.46 and 50 mg/L over period of 72 hrs. Aminobenzonitrile degraded by the microbial consortium follows the NHase degradation pathway based on the product, 2-piperidinone (cyclic amide) detected. Based on the results of the study the hypothesis is accepted, microorganisms isolated from Au mine tailings are able to degrade organic contaminants namely aminobenzonitrile.

5.2 Recommendations

As this study focused on the biodegradation of benzonitrile by bacterial consortium isolated from Au mine tailings. The recommendations for future studies are:

- The study was conducted on a superficial media (M.M), therefore the next step would include running degradation studies on actual contaminated sites or actual contaminated media samples.
- Biodegaradation and optimisation studies should be conducted based on the other four classes of contaminants identified namely: ethers, pyridines, pyrimidines and phenols.
- The reaction mechanisms of the biodegradation of the above mentioned contaminants by the bacterial consortium.
- Isolate and identify culturable fungi species from the Au mine tailings and study their ability to biodegrade benzonitriles as well as the four classes of identified environmental contaminants.
- Study the reaction mechanism/pathway of the biodegradation of each contaminant class by the isolated fungi.

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Appendix A:

Bacteria was isolated from the Au mine tailing samples and the genomic DNA was extracted from the isolated bacterial samples. The 16S rDNA target region was amplified via PCR and the PCR products of 16S region that were ran on agarose gel is depicted in Figure 7.1.

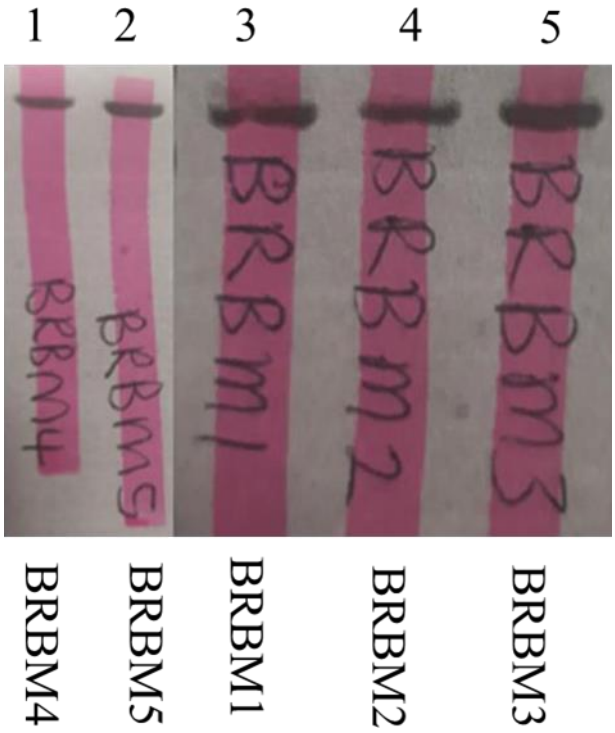

Figure 7. 1: Agarose gel containing the PCR products of the 16S gene found in pure bacterial samples.

Lanes 1 – 5 represents the samples; BRBM4, BRBM5, BRBM1, BRBM2, and BRBM3 respectively.

Biodegradation tests were done under varying conditions as indicated in the materials and methods section. Samples were taken from initial and final runs of the biodegradation tests and analysed on GC-MS. Figure 7.2 (appendix) indicates a sample of GC-MS data acquired for the standard 500 mg/L used to construct the standard curve graph. A standard curve was constructed on GC-MS, using known concentrations of aminobenzonitrile

(figure 7.3) as standards, and the concentration of aminobenzonitrile for both the initial (Ci) and final (Cf) experiments (runs) were extrapolated via quantitative analysis through GC-MS (table 4.9). Table 4.9 also indicates the biodegradation % of each experiment (run).

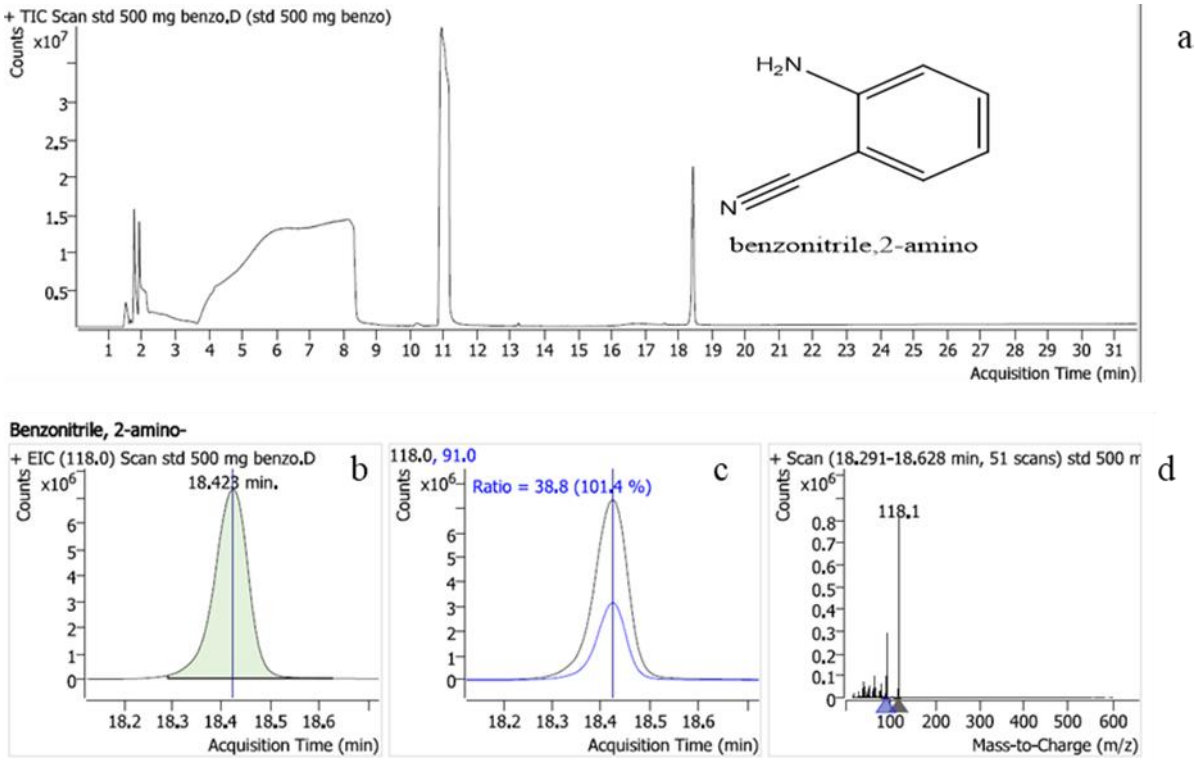

Figure 7. 2: GC Chromatogram for standard concentration of 500 mg/L aminobenzonitrile in M.M (a), (b) indicates the peak for benzonitrile,m-amino at 18.423 min, (c) ratio of fragmentation of two dominant peaks used to identify benzonitrile,m- amino, (d) scanned hits/fragments obtained for benzonitrile,m-amino.

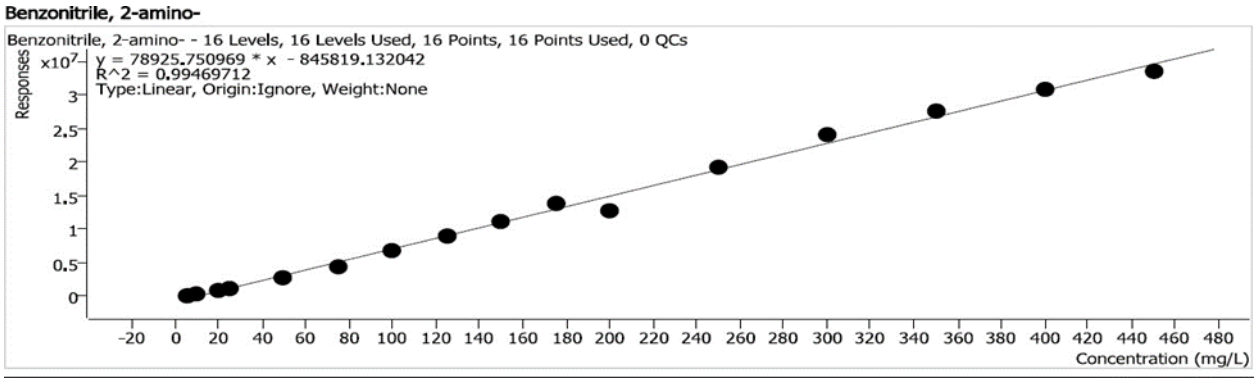

Figure 7. 3: Standard curve constructed from known concentrations of aminobenzonitrile ranging from 5 mg/L to 500 mg/L.

Figure 7.3 illustrates the standard curve obtained from GC-MS analysis of known concentrations of aminobenzonitrile (Benzonitrile,m-amino-), 16 concentrations were used to plot the graph ranging from 20 mg/L to 500 mg/L. A regression value (R2) of 0.99469712 was obtained indicating the standard curve (line of best fit) is well represented in the graph. The biodegradation percentage (%) of each experiment is also shown in table 4.9.