OPTIMIZATION OF ELEMENTAL SULPHUR RECOVERY DURING ACID MINE WATER TREATMENT

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ABSTRACT

Title: Optimization of Elemental Sulphur Recovery during an Acid Mine Water Treatment

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The South African mining industry is a major contributor to South Africa's Gross Domestic product (GDP). The negative consequences of mining include toxic effluents from mineral processing and decanting streams, even after mine closure. Large volumes of Acid Mine Drainage (AMD) are expected to increase as the mining industry grows. Currently, biological treatment of mine waters are preferred to chemical methods, due to various advantages offered such as low operational cost and small environmental footprint. Biological treatment of AMD primarily rely on the activity of sulphate reducing bacteria which reduce sulphate to sulphide in the presence of organic matter thus allowing the precipitation of the metals and increase in pH. However, excess of sulphide remains in the system and if not removed, can be oxidized to sulphate.

A sustainable AMD management plan could entail development of treatment technologies to remove total sulphur (sum of sulphur species) from the system. Production of elemental sulphur, which involves partial oxidation of sulphide, has been a recent subject of interest. The use of colourless sulphide oxidizing bacteria, especially *Thiobacillus species* has been widely reported.

Six isolates of sulphide oxidizing microorganisms, of which 4 bacterial and 2 filamentous fungal species from a gold mine (Johannesburg, South Africa) were tested in this study to achieve partial oxidation of sulphide to sulphur. The microbial species were selected for high sulphide oxidation in the presence of carbon sources (glucose and lactate). *Lysinicibacillus fusiformis* was observed to be the most suitable microorganism for sulphide oxidation. In

order to investigate optimal conditions for sulphur recovery, *L. fusiformis* bacterial activity was tested under different conditions of pH and redox potential. It resulted that at a pH of 8 and Eh of -80mV up to 95% of sulphur was recovered.

DECLARATION

I Francine Dimite Alesia, declare that the thesis which I hereby submit for a Master of Science in Environmental Technology degree at the University of Pretoria is my own work and has not been previously submitted by me for any degree at this or other institutions.

_____ _____

Francine Dimite Alesia Date

_____ _____

Evans M.N. Chirwa Date

(Promotor)

Dedicated to:

The MOUDIO MONEY Family

A family who teaches me every day about God, Love, Unity and Family values.

EYINDO MOUYEMA MARIE-THÈRÈSE

For the seed of faith that grows in me,

and;

PRISO KOTTO ISIDORE JOAQUIM

My Hero.

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LIST OF ABBREVIATIONS

AMD	Acid mine drainage
BLAST	Basic local alignment search tool
bp	Base pairs
COD	Chemical oxygen demand
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
Eh	Redox potential
EPA	Environmental Protection Agency
HPLC	High performance liquid chromatography
ICP-OES	Inductively coupled plasma optical emission spectropscopy
M	Molarity
MB	Methanogenic bacteria
SA	South Africa
SEM	Scanning electron microscopy
SOB	Sulphide oxidizing bacteria
SOM	Sulphide oxidizing microorganisms
SRB	Sulphate reducing bacteria
TEM	Transmission electron microscopy

CHAPTER 1

INTRODUCTION

1.1 Background

The mining industry represents the largest income generating activity for South Africa. Mining operations in South Africa focus on the extraction principle of precious minerals such as gold, as well as fossil fuels like coal. Coal is one of the sources of energy in South Africa, dominating other energy sources by up to 77% and serves as the region's main source of electricity (Department of Energy South Africa). Coal mining also contributes towards the national automobile fuel supply, with more than 40% of fuel coming from the Sasol synthetic fuel process based on coal as its feed stock (Department of Energy South Africa). Mining activities in South Africa have led to an inevitable occurrence of Acid Mine Drainage (AMD). AMD negatively impacts on soil and water in regions where mines are situated. It is an environmental ill-effect characterised by low pH, high sulphate concentration (often referred to as high salinity) and high concentrations of heavy metals (McCarthy, 2011; Pentreath, 1994; Jenkins *et al.* 2000). These characteristics make AMD a hazardous phenomenon that should be urgently addressed (Ochieng *et al.*, 2010).

There have been various attempts to mitigate the effects of AMD on the environment. Physical and chemical treatment methods have been explored, but have been considered less advantageous compared to biological methods (Johnson *et al.*, 2005, Sahoo *et al.*, 2013; Johnson & Hallberg, 2005; Neba, 2006). Biological treatment of AMD relies on the use of Sulphate Reducing Bacteria (SRB), which are capable of reducing sulphate to sulphide in the presence of organic matter. The activities of these bacteria lead to the accumulation of bicarbonate as a by-product. The bicarbonate produced increases the pH of affected water, which results in the precipitation of heavy metals from solution (Greben *et al.*, 2005; Cao *et al.*, 2009; Tang *et al.*, 2009, Singh *et al.*, 2011). Sulphide at medium and low pH leaves the solution as the gaseous hydrogen sulphide (H_2S) which as a strong smell and is corrosive to metallic surfaces (Celis-Garcia *et al.*, 2007). For these reasons an effort is made to remove sulphide from effluents from sulphate reduction processes as a means to reduce environmental impacts.

Studies that focused on the removal of sulphide from AMD include precipitation as metal sulphide (Dvorak et al., 2004), oxidation to elemental sulphur (Janssen et al., 1999; Rein, 2002; Molwantwa, 2008 and Mooruth, 2011), solvent extraction (Janssen et al., 2000 and Johnson, 2000) and electrochemical oxidation (Waterson et al., 2006). The partial oxidation of sulphide to produce elemental sulphur is of great interest because the produced elemental sulphur could be used as fertilizers, as a substrate for the bioleaching processes and as raw material for production of plastics and fire retardants (van Lier *et al.*, 2001; Celis-Garcia *et al.*, 2008). Other studies have attempted to optimize the production of elemental sulphur by manipulating biological pathways in sulphide oxidizing bacteria (Reinhoudt & Moulijin, 2000; Rein, 2002; Molwantwa, 2008; Krishnakumar *et al.*, 2005; Cardoso *et al.*, 2006; Lee *et al.*, 2006). In spite of the above efforts, a lot is still unknown about the optimal conditions for elemental sulphur production, hence the need to investigate the possibility of enhancing elemental sulphur production.

1.2 Objectives of Study

The production and recovery of elemental sulphur via the use of Floating sulphur Biofilms during AMD treatment has been highlighted to be of great importance previously (Rein, 2002, Neba *et al.*, 2006, Molwantwa, 2008; van Hille & Mooruth, 2011, Rose *et al.*, 2013), using Biotrickling filter reactors mentioned by Fortuny et al., 2009 and 2011) or the expanded granular sludge bed reactor (Chen et al., 2008). However, optimal conditions for the process remain unknown. The primary objective of this study is to investigate the optimal conditions for simultaneous acid mine water treatment and partial sulphide oxidation to produce and recover elemental sulphur. It was imperative, during the course of the study, to monitor key parameters such as temperature, redox potential and pH to determine optimal values that could positively influence the production of elemental sulphur.

1.3 Outline of Dissertation

This dissertation includes:

- Chapter 1- Introduction provides a brief background of the AMD problem and its various treatments are given with a highlight of the importance to produce elemental sulphur during the treatment.

- Chapter 2- Literature Review gives sufficient background information regarding previous attempts to optimize elemental sulphur production and recovery. The chapter also covers studies that have focused on the occurrence of different sulphur species in water, the impact of sulphur species pollutants present in water, on living organisms and the environment, the different remediation strategies that have been explored as well as identified species of sulphide oxidizing microbes and the biological pathways of the sulphur oxidizing process.

- Chapter 3- Materials and Methods explains the research methodology including details on the analytical methods employed for successful completion of this study.

- Chapter 4- Culture characterization and performance analysis explains the identification and characterization of microbial species as well as their ability to oxidize sulphide.

- Chapter 5- Kinetic Modelling theory describes the use of an appropriate kinetic model performed in order to determine the optimum conditions for sulphur recovery.

- Chapter 6- Conclusion and Recommendations represents a summary of the findings and conclusions of the work performed in the study and recommendations are given for further study.

CHAPTER 2

LITERATURE REVIEW

2.1 Acid Mine Drainage

2.1.1 The Sulphur Cycle

Sulphur is the tenth most common element on earth. It is found in proteins and vitamins and several organic constituents. Plants take up dissolved sulphur, while animals consume plants to obtain sufficient sulphur to maintain their health. In the natural environment, sulphur is mostly stored in sedimentary rocks and salted sea. It can enter the atmosphere through natural processes (volcanic eruptions, bacterial processes and evaporation) or industrial processes (Figure 2.1).

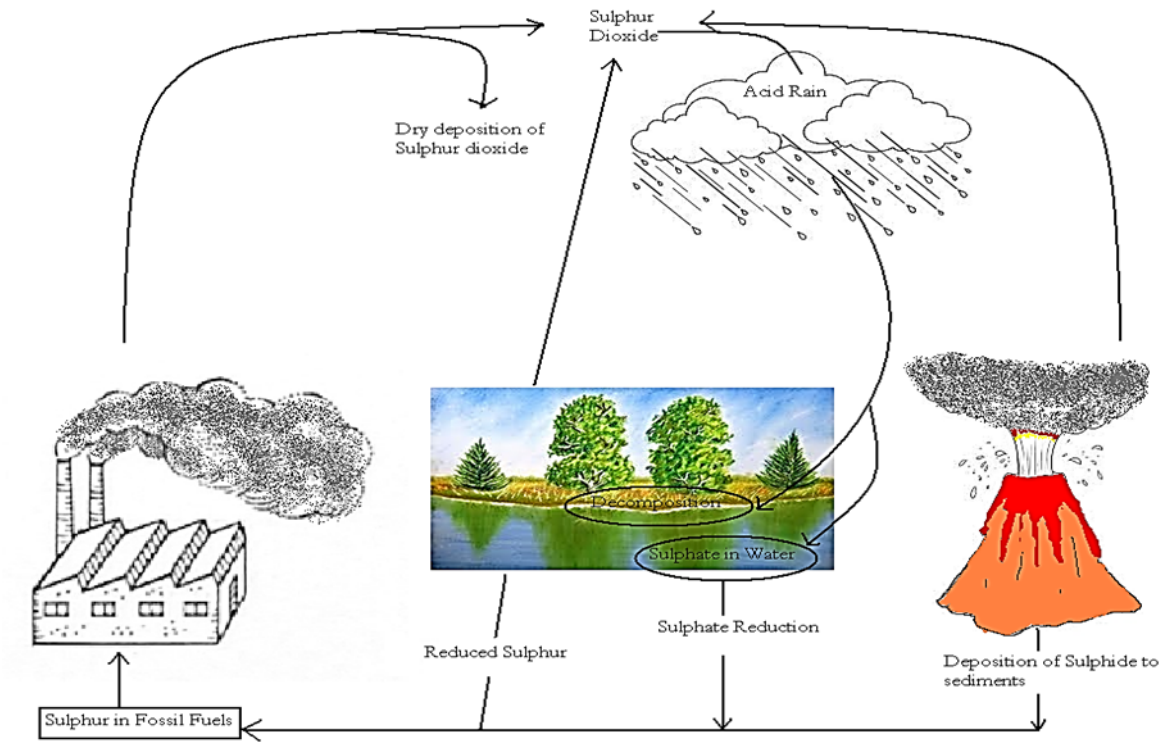

Figure 2.1: Sulphur Cycle in the Environment

The natural sulphur cycle maintains the balance of distribution of elemental sulphur in the global environment. Unfortunately, human activities tend to disrupt the natural cycle in the environment, by increasing the concentration of hazardous sulphur species in certain environmental compartments to levels that may be detrimental to indigenous organisms. Sulphur dioxide (SO_2) is one of the hazardous sulphur species released into the environment due to combustion of fossil fuels such as coal, and is generated from activities also producing acid mine drainage. SO_2 generated during combustion of coal is easily oxidized in the atmosphere and protonates to form sulphuric acid upon contact with atmospheric water. Sulphuric acid (H_2SO_4) can further react with other compounds to form di-methylsulphide which is eventually emitted to the atmosphere by plankton species. These particles are either deposited on earth or react with the rain to form acid deposition, also known as acid rain.

2.1.2 Formation of Acid Mine Drainage

The presence of sulphide minerals, particularly pyrite (FeS_2) and their oxidation products drive the occurrence of AMD. Nordstrom & Alpers (1999) reported that pyrite concentration, grain size and its availability affect the generation of AMD. In the presence of oxygen and water, pyrite dissociates into ferrous ions (Fe^{2+}) and sulphide (S^{2-}), which are readily oxidized to sulphate ions (SO_4^{2-}) (Equation 2.1). The oxidation process includes production of hydrogen ions (H^+), which contribute to the acidity of the solution.

$$2FeS_2+7O_2+2H_2O \rightarrow 2Fe^{2+} + 4SO_4^{2-} + 4H^+ \quad (2.1)$$

The ferrous ions (Fe^{2+}) produced in Equation 2.1 are further oxidized by oxygen to release Ferric ions (Fe^{3+}) (Equation 2.2). The Fe^{3+} ions are hydrolysed to produce an insoluble compound ferric hydroxide [$Fe(OH)_{3(s)}$] (Equation 2.3).

$$4Fe^{2+}+O_2+4H^+ \rightarrow 4Fe^{3+}+2H_2O \quad (2.2)$$

$$Fe^{3+}+3H_2O \rightarrow Fe(OH)_{3(s)}+3H^+ \quad (2.3)$$

The reaction (Equation 2.3) shows a net production of 3 moles of H^+ per mole of pyrite oxidized. Owing to this, the pH of the solution drops to create a highly acidic environment, which is characteristic of AMD.

$$FeS_2+14Fe^{3+}+8H_2O \rightarrow 15Fe^{2+}+ 2SO_4^{2-} +16H^+ \quad (2.4)$$

Several factors have been highlighted as crucial to the occurrence of AMD. These include the presence of iron oxidizing and sulphide oxidizing bacteria, the temperature, oxygen content and sulphate concentration as well as concentration of heavy metals (Valenzuela *et al.* 2005).

Thus AMD is characterized by:

- Low pH
- High Sulphate concentration (or salinity)
- High metal content

2.1.3 Impacts of Acid Mine Drainage

There are severe adverse effects that occur due to AMD one of which is the pollution of receiving water bodies-both surface and groundwater. The discharge of AMD into natural water bodies increases the acidity of the receiving waters and the resulting acidity negatively impacts animal and plant life in natural ecosystems (Riley *et al.*, 1972). Jamal (1997) indicated that the effect of AMD is not restricted to the local area at the source but may extend further if the acid water is discharged into a stream or river without adequate treatment. If acid producing mines are located in regions with permeable soil structures, the polluted acid mine water could percolate into the aquifers and spread over a wide area through groundwater movement. It has also common knowledge that acid generation and discharge still occurs long after a mine is closed or abandoned (Lottermorser, 2003).

The pollution of groundwater is not the only hazard that AMD poses. A study by Atkins and Singh (1982) showed that acidic water was responsible for the corrosion of equipment at mines. AMD accelerates the formation of scales in delivery pipes as well as the pollution of the mine surface environment, affecting surface ecology. AMD can inhibit or kill some aquatic plants and animals thereby causing undesirable ecological shifts. Low pH values, such as those experienced in regions affected by AMD could cause respiratory or osmo–regulatory

failure in fish and low order vertebrates (Kimmel, 1983). At low pH levels, hydrogen ions may be absorbed by cells displacing vital sodium ions (Morris *et al.*, 1989), which are important for normal body operation. Ferric hydroxide [$Fe(OH)_{3(s)}$], commonly known as Ochre (Equation 2.3), is one of the by-products of AMD. It is a low density compound with an orange colour and is often found at the bottom of affected rivers. The presence of $Fe(OH)_3$ disrupts the food supply for benthic organisms leading to their death and disruption of the aquatic food chain. It has also been reported that ochre reduces the surface area available for fish to lay their eggs thereby affecting their production cycle (Pentreath, 1994).

AMD is known to be a serious threat to humans and ecological systems due to the presence of heavy metals. Heavy metals cannot be degraded; they mostly accumulate higher in the food chain (Moreno *et al.*,2001 and Carlson *et al.*, 2002). The low pH of the mine water increases solubility of the heavy metals in water thereby increasing the concentration of the metals and their toxicological effects on aquatic ecosystems. According to the study done by Lewis and Clark (1996), exposure of higher order organisms to high concentrations of heavy metals results in acute effects such as stunted growth, lower reproduction rates, deformities and increased mortality. For example, in fish, acidity from AMD may cause various physiological disturbances such as reduced growth and reproduction rates (Kimmel, 1983). Furthermore, the high concentration of heavy metals in affected water has been reported to affect algal growth. Algae are the primary producers in aquatic systems and suppression of their growth rate affects the proliferation of aquatic life (Hoehn and Sizemore, 1977). Direct input of ochre in fish population includes blockage of gills and suffocation of eggs which could drastically reduce the fish population (Hoehn and Sizemore, 1977).

Aluminium is one of the metals found in water bodies whose organisms and speciation in water bodies can be affected by AMD. Significant amounts of aluminium in water combined with a low pH increases the rate of sodium loss from blood and tissue, resulting in death. Brown and Sadler (1989) showed that the loss of sodium ions from blood resulted in high mortality in fish and other aquatic organisms in water bodies that were polluted by AMD. Additionally, the precipitation of aluminium in fish gills affects their breathing. Earle and Callaghan (1998) observed that receiving waters contaminated by AMD had low biodiversity.

The effects of iron precipitates are similar to those of aluminium precipitates, i.e., they form a blanket at the stream bottom, adversely affecting both macro-invertebrates and fish.

2.2 Production of Sulphide

Different mechanisms including sulphate reduction are known to produce sulphide (Figure 2.2) and industries such as tanneries as well as paper and pulp manufacturers are also known to generate sulphide (Janssen *et al.*, 1999).

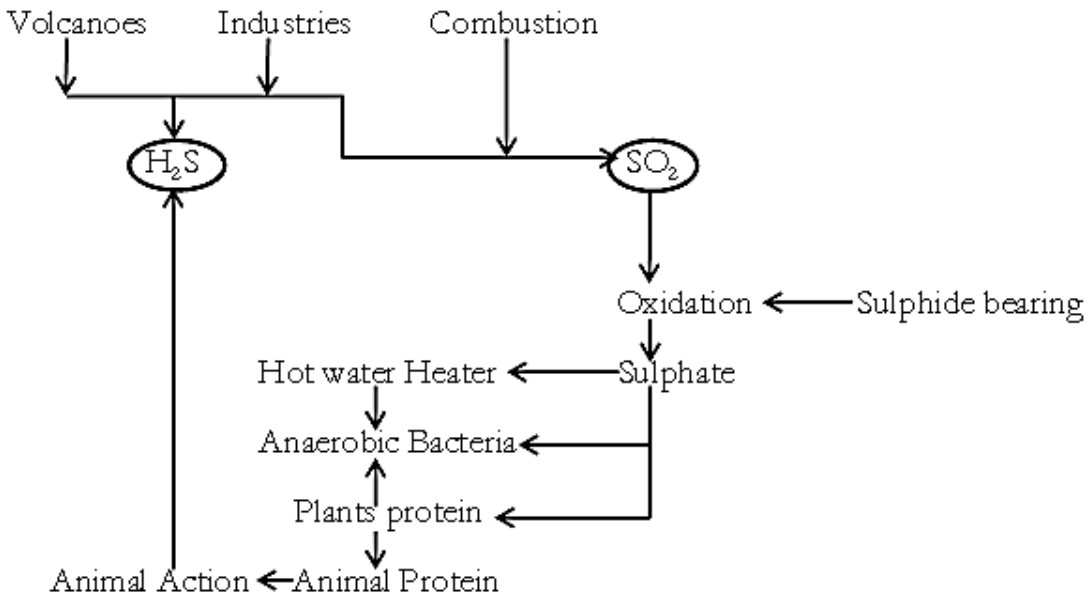

Figure 2.2: Various mechanisms generating hydrogen sulphide (Modified from Edwards et al, 2011)

Sulphide is mostly present in three different forms, depending on the pH of the liquid medium in which it is found (Figure 2.3). The three forms in which sulphide is found are:

- Hydrogen sulphide (H_2S) - mostly dominant at pH 5-6
- Bisulphide ion (HS^-) - recurrent at pH 7-9
- Sulphide ions (S^{2-}) - dominant at a pH>9

The sum of the three compounds above is referred to as total sulphide expressed as a concentration with the units mg/L S^{2-}. The existence of other forms of sulphide also depends on the oxidation reduction potential (ORP) as shown in Figure 2.4.

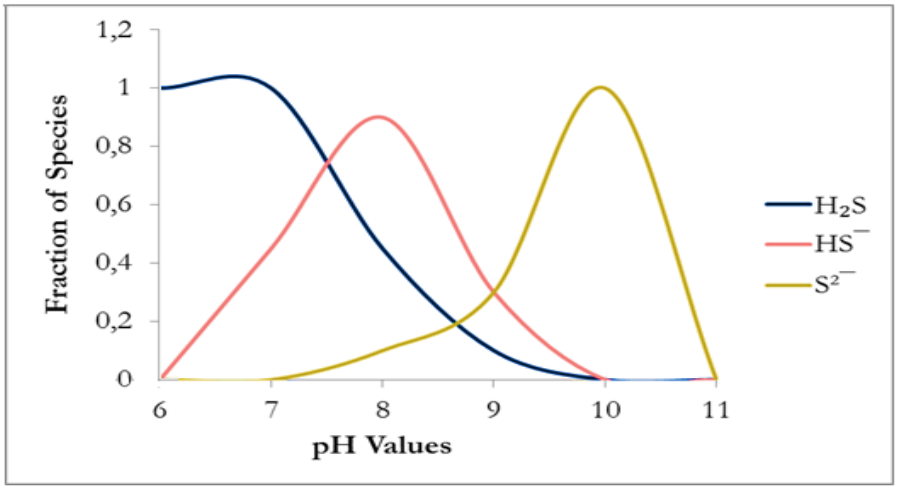

Figure 2.3: Ionic species of hydrogen sulphide at different pH (Modified from Thompson *et al.*, 1995)

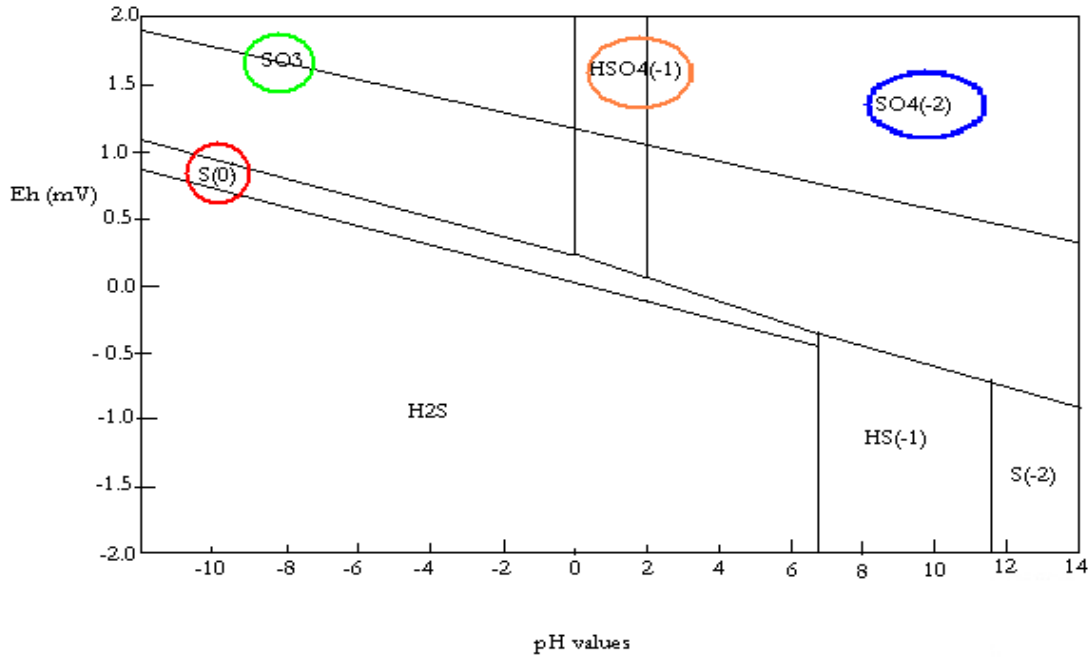

Figure 2.4: Sulphur species at different pH values and Redox potential (Eh) values (Modified from Kriek et al., 2013)

2.2.1 Physiological and Environmental Effects of Sulphide

The rotten smell egg of hydrogen sulphide renders contaminated water undesirable for drinking and if ingested stomach discomfort, nausea and vomiting will occur (Health Canada, 1992). However sulphide poisoning mostly occurs via inhalation: Chou (2003) explained that an average individual can detect the presence of hydrogen sulphide at 11µg/m^3 but at higher concentration (140-700 mg/m^3) it will be fatal. Table 2.1 summarises the various health effects that could result from short-term exposure to sulphide.

Table 2.1: Health effects from short-term exposure to hydrogen sulphide (Adapted from Skrtic, 2006)

Concentration (ppm)	Health effect
0.01-0.3	Odour threshold
1-20	Offensive odour, possible nausea, tearing of the eyes or headaches with prolonged exposure
20-50	Nose, throat and lung irritation; digestive upset and loss of appetite; sense of smell starts to become fatigued; acute conjunctivitis may occur (pain, tearing and light sensitivity)
100-200	Severe nose, throat and lung irritation; ability to smell odour completely disappears.
250-500	Severe lung irritation, excitement, headache, dizziness, staggering, sudden collapse, unconsciousness and death within few hour, loss of memory for the period of exposure resulting in permanent brain damage if not rescued immediately
500	Respiratory paralysis, irregular heartbeat, collapse and death without rescue
>1000	Rapid collapse and death

Sulphide is also associated with corrosion problems to drill strings in mining operations, transport pipes or drainage pipes and effluent storage pipes. These problems increase the operation and maintenance cost of plants handling H_2S contaminated water. According to a report by Lyn (1992), hydrogen sulphide removal could be achieve using an oxidative method but this method leads to increased turbidity and colour in the effluent water.

2.3 Treatment of Acid Mine Drainage

The systems employed in the treatment of acid mine drainage can be classified as either active or passive systems, depending on whether the system requires energy or labour inputs during its operation. A range of configurations incorporating both physical/chemical and biological processes have been successfully employed in both passive and active processes. Various factors influence decision making during the selection of a treatment system. Figure 2.6 shows the decision tree for selection between active and passive systems. Many active mining companies prefer active systems since the cost of treatment process is covered by the

profits of the company. On the other hand, budget limitations towards remediation operations at closed mines, favours the implementation of passive systems.

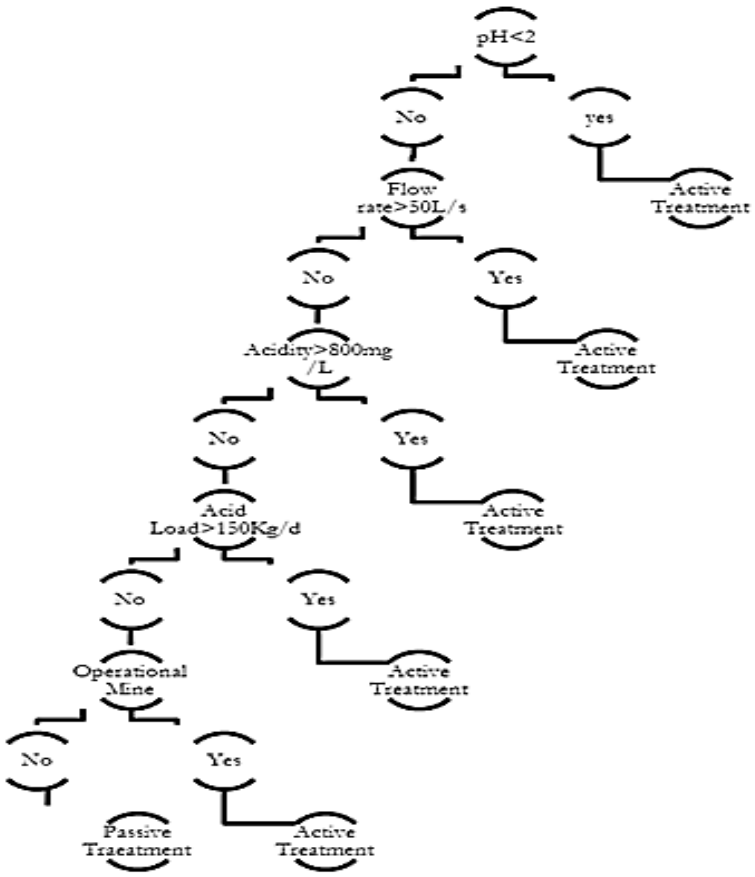

Figure 2.5: Decision flow chart for selection between active and passive treatment of AMD (Modified from Waters et al., 2003)

2.3.1 Passive Treatment Systems

Passive treatment refers to a treatment process where human intervention is minimal or absent. Pulles et al (2004) defined passive system as a water treatment system that uses natural available energy sources such as topographical gradient, microbial metabolic energy, photosynthesis and chemical energy. Some of the processes require a regular but infrequent maintenance to operate successfully throughout their lifespan. Examples of passive systems that have been used in treatment of AMD are shown below in Figure 2.7. Natural systems such as aerobic and anaerobic wetlands are utilized for removing organic matter where as

physical chemical processes such as Alkalinity Producing Systems (APS), Anoxic Limestone Drains (ALD) and Limestone ponds are suitable for the precipitation of metals in AMD.

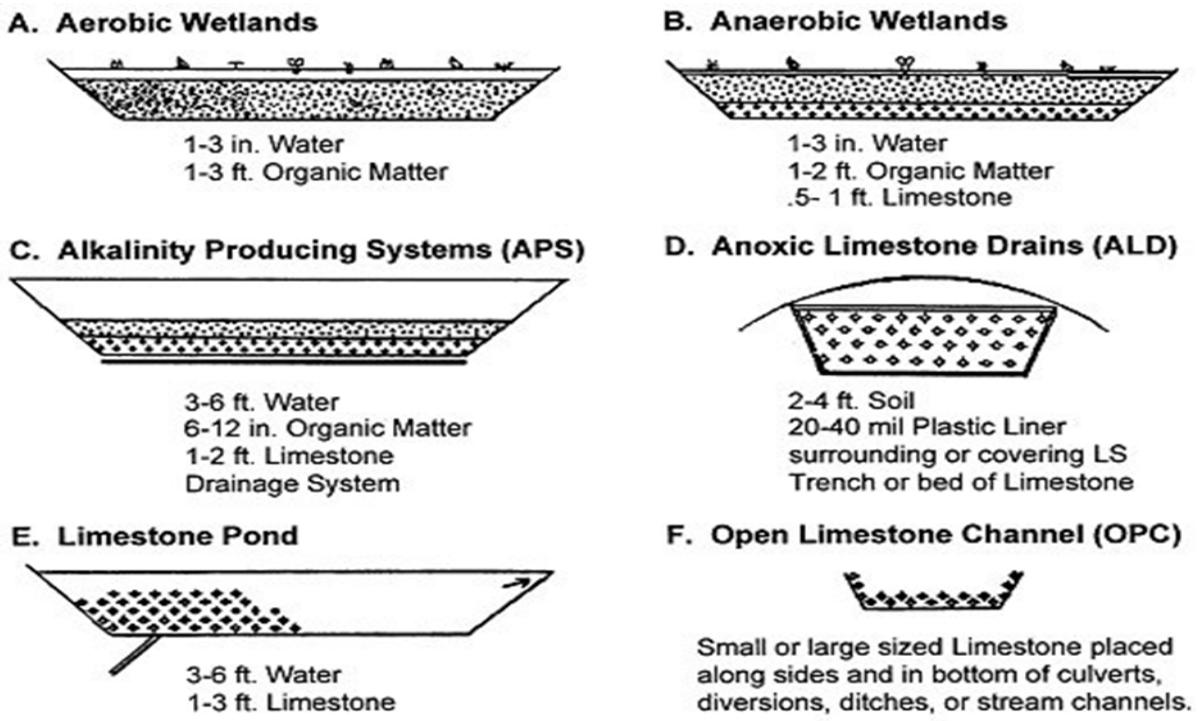

Figure 2.6: Different Passive Treatments (Skousen et al., 1998)

A specific example of a decision to choose between the different passive treatments towards specific water quality goals is shown in Figure 2.8 based on the process proposed by Herdin et al. (1994). This decision diagram was later modified by the Piramid consortium (2003) and later by Gusek (2008) who updated it in in order to include a wider range of chemical elements since previous versions were only focused on iron and magnesium.

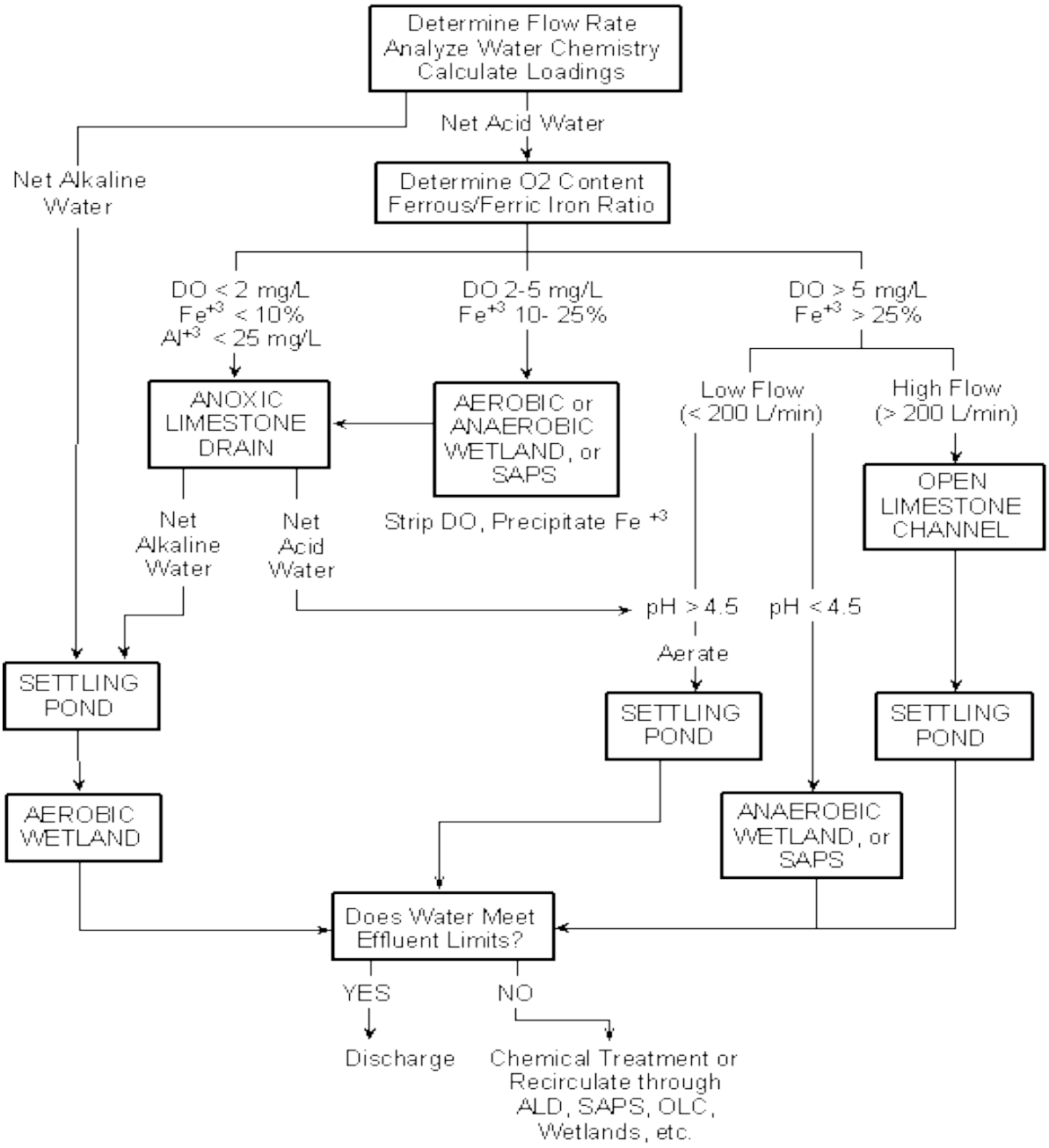

Figure 2.7 Decision Tree for selection the passive system to treat AMD (Adapted from Herding *et al.*, 1999).

2.3.1.1 Examples of physic-chemical processes used as passive systems

(a) Anoxic Limestone Drains (ALD)

In the anoxic limestone drain (ALD) system, acid mine drainage is allowed to flow over crushed limestone cells. The cells are sealed at the point of discharge of AMD to minimize oxygen ingress and to ensure that carbon dioxide (CO_2) accumulates in the system. In an earlier study by Hedin and Watzlaf (1994), ALDs were capped with clay to prevent contact with oxygen. Perforated plastic sheets are sometimes placed between the cells to prevent clay and dirt from entering the cells. The limestone dissolves in the acid water, raising the pH to around 7 (Equation 2.5). During this process, iron is maintained in its reduced form, thus inhibiting the formation of ferric hydroxide, which could reduce the effectiveness of the ALD system (Johnson and Hallberg, 2005).

$$CaCO_3+H^+ \rightarrow Ca^{2+}+HCO_3^- \quad (2.5)$$

Although, an increase in pH is achieved at low cost in ALDs, the system is susceptible to the coating by ferric and aluminium precipitates. Another shortcoming is the formation of ferrous carbonate and manganous carbonate gels within the limestone which causes fast dissolution of the limestone pebbles (Evangelou, 1998).

(b) Open Limestone Drains (OLD)

Open limestone drains (OLDs) are designed to increase pH through dissolution of exposed limestone surface in specially constructed limestone drains. The design and operation of the system demand special attention during the armouring and coating of the limestone drains. Ziemkiewicz et al. (1997) utilised this system and observed that the OLDs were efficient in removing iron and increasing pH. In spite of the feasible performance observed, the increase in pH was eventually hampered by a ferric iron coating that developed during operation in the long-term. Construction with steeper gradients for high flow velocities could alleviate this problem. The OLDs can also be periodically flushed to remove any accumulated precipitates.

2.3.1.2 Examples of Biological Processes uses as a Passive Systems

Wetlands are usually the favourable option for use as passive treatment of AMD mainly because they are relatively self-sustainable once they have been established, and they can be

constructed and operated at a low cost. Designed constructed wetlands are used in the treatment of AMD although natural wetlands have also been integrated in AMD treatment schemes (Johnson and Hallberg, 2002).

a) Aerobic Wetlands

Aerobic wetlands are primarily used when iron is the main contaminant. They are usually shallow, and include plants that are used to immobilise heavy metals. The following operational conditions are used to maintain aerated conditions on the wetland:

(1) Relatively shallow water depths to allow aeration of the mine drainage
(2) Cascades to further enhance aeration
(3) Sufficient residence time to allow the treatment reactions to take place
(4) Space for the settling and accumulation of the metal precipitates and solids
(5) Promote algal growth to further increase the pH and facilitate manganese oxidation and precipitation

Items 3 and 4 require large land areas and pose a challenge for mines in operation (Skousen et al., 1998). A drawback of aerobic wetlands is the accumulation of precipitates over time that will limit their abilities to treat the acid mine waters (Costello, 2003).

b) Anaerobic Wetlands

Anaerobic wetland systems depend on abiotic and biotic reactions to precipitate metals and neutralize acidity. The ability of these wetlands to generate alkalinity passively makes them suitable for treatment of discharge from abandoned mines. The reduction reactions that occur within the wetlands are driven by electron donors that originate from organic matter present in the environment. There are two types of anaerobic wetlands namely: (1) a basic wetland where dissolved oxygen which is of 2-5 mg/L in the acid water (see Figure 2.6) is replenished thereby promoting the reduction of iron and sulphate, and (2) a variant anaerobic wetland, which is the combination of a basic anaerobic wetland and the addition of limestone gravel to produce more alkalinity. The bicarbonate produced from the addition of limestone helps to neutralize the acidity of AMD by increasing the pH and subsequently, the precipitation of metals. A drawback for the system is the slow mixing of the alkaline

substrate water with AMD near the surface. This can be overcome by constructing very large wetlands.

2.3.2 Active Treatment Systems

Active systems refer to systems that require constant human intervention, operation or maintenance and monitoring, and require a constant supply of energy mostly in the form of heat or electricity. Table 2.2 list the shortcomings and advantages of active treatment systems.

Table 2.2: Advantages and Disadvantages of Active Treatment (Adapted from Motsi, 2010)

Advantages	Disadvantages
Effective and fast removal of acid and metals	High initial capital costs
Frequent process monitoring	High chemical costs
Precise process control	High operational costs
Can be accommodated at small sites	Disposal of the sludge

2.3.2.1 Examples of physic-chemical systems used as Active Systems

Active abiotic systems involve the addition of a chemical agent that will raise the pH resulting at the acceleration of the chemical reactions rates so promoting metal precipitation through the formation of hydroxides or carbonates. Coagulants can be added to facilitate settling and mechanisms for the removal of resultant sludge are included. An example of a decision to choose among the various active treatments towards specific water quality goals is shown in Figure 2.8.

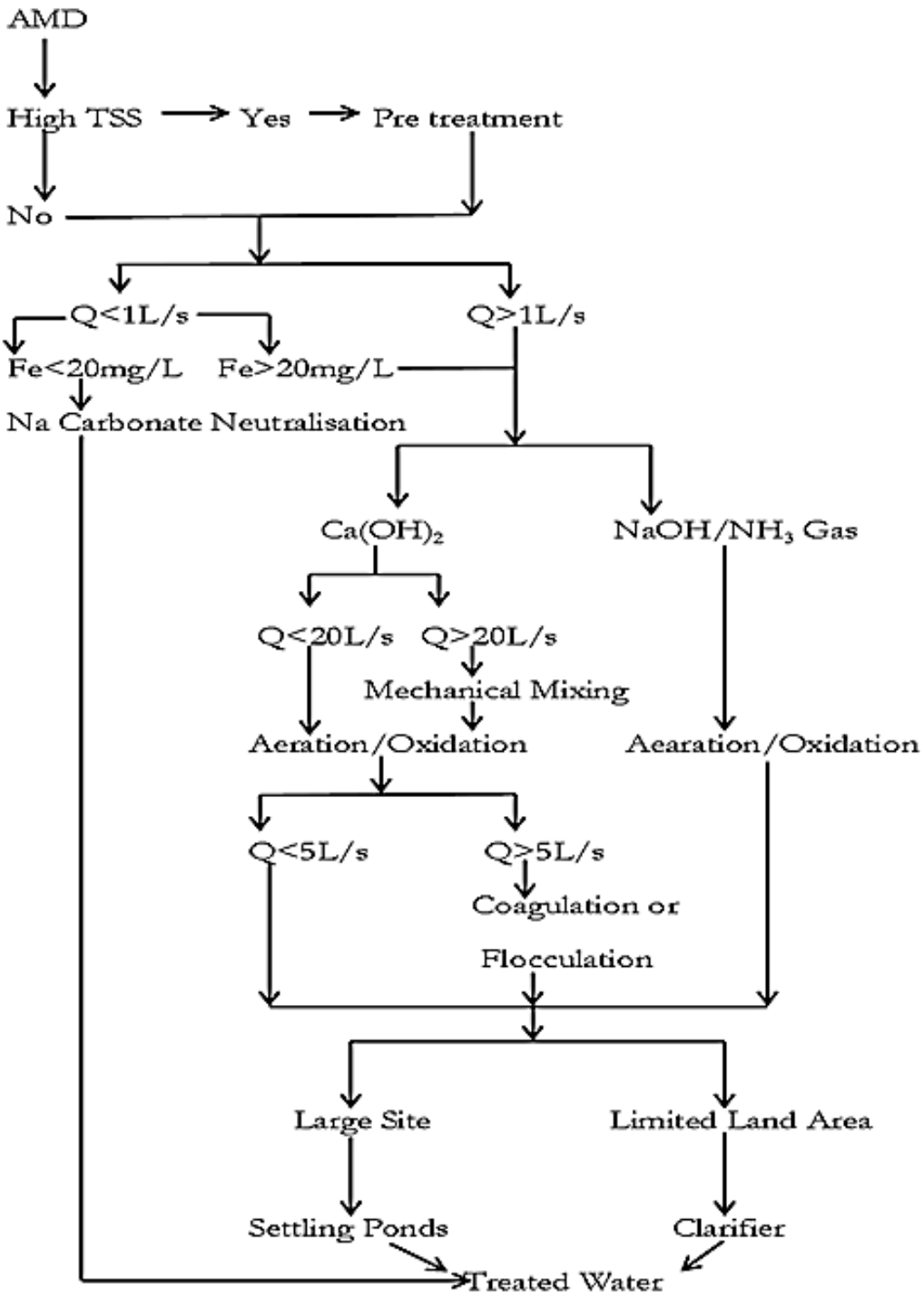

Figure 2.8: Decision Tree for selection of the active treatment systems (Modified from Rajaram et al., 2001).

Table 2.3: List of Chemicals used in Active treatment (Adapted from Motsi, 2010)

Name	Comments
Oxidants	
Calcium Hypochlorite ($Ca(ClO)_2$	Strong oxidant
Sodium Hypochlorite (NaClO)	Strong oxidant
Calcium Peroxide (CaO_2)	Acid neutraliser
Hydrogen Peroxide (H_2O_2)	Strong oxidant
Potassium Permanganate ($KMnO_4$)	Very effective and commonly used
Acid Neutralisation	
Limestone ($CaCO_3$)	Used in anoxic limestone drains and open limestone channels
Hydrated Lime ($Ca(OH)_2$)	Cost effective reagent, requires mixing
Pebble Quick Lime (CaO)	Very reactive, needs metering equipment
Soda Ash Briquette (Na_2CO_3)	System for remote locations but expensive
Caustic Soda (NaOH)	Very soluble, can be either in solid or liquid form. It is cheaper in liquid form
Ammonia (NH_3)	Very reactive and soluble
Fly Ash ($CaCO_3$, $Ca(OH)_2$	Neutralisation value varies with each products
Coagulants/Flocculants	
Alum ($Al_2(SO_4)_3$	Acidic material, forms $Al(OH)_3$
Copperas ($FeSO_4$)	Acidic material, usually slower reacting than alum
Ferric Sulphate ($Fe_2(SO_4)_3$)	Ferric products react faster than ferrous
Sodium Aluminate ($NaAlO_2$)	Alkaline coagulant

2.3.2.2 Examples of Biological Systems Used as Active Systems

Active biological systems require continuous direct intervention in their operations. Due to their high performance, they are usually chosen over passive systems in mines operations, which are still open. Active biological systems rely on the activity of sulphate reducing bacteria (SRB) that reduce sulphate to sulphide and ferrous iron oxidizing bacteria that facilitate the removal of iron as iron hydroxide precipitate. Examples of active biological systems that have been implemented successfully include activated sludge and floating sulphur biofilms processes.

(a) Activated Sludge with Sulphate Reduction

Ingvorsen *et al.*, (2003) demonstrated that sulphate reducing bacteria (SRB) can remove sulphate in an activated sludge plant under anaerobic conditions. Bade *et al.*, (2000) and Cypionka (2000) however reported that some SRB strains can tolerate oxygen while other strains could use oxygen as an electron acceptor for the production of adenosine triphosphate (ATP).

(b) The BioSURE Process

The Rhodes BioSURE process was developed in Grahamstown, South Africa in the early 1990s in collaboration with the Council for Scientific and Industrial Research (CSIR) (Rose, 1992, Rose *et al.*, 1996 and Dunn, 1998). The process utilizes chemical oxygen demand (COD) in sewage sludge as an electron donor or carbon source for the sulphate reduction by microbes. The sustainability of the BioSURE process is dependent on a variety of factors, some of which were listed by Neba (2006). Some of these factors are explained below:

(1) *Environmental sustainability*- the process includes the removal of sulphate present in AMD and the disposal of primary sewage sludge. This will benefit a water scarce country such as South Africa.
(2) *Technical sustainability*- Low cost of technical setup.
(3) *Financial sustainability*- Low cost of the process due to readily available and affordable carbon sources.
(4) *Social Sustainability*- Kumalo (2005) and Rose et al (2009) emphasized the fact that the treatment of AMD could create employment, hence alleviate poverty.

In spite its advantages, only pilot studies have been conducted using BIOSURE process.

(c) Floating Sulphur Biofilms

The use of floating sulphur biofilms to treat acid mine drainage has been a subject of interest in the development of a bacterial system. The appearance of white films (biofilms) on sulphate reducing systems had been observed in the past (Jørgensen & Revsbech, 1985; Janssen *et al.*, 1997; Rose *et al.*, 1996; Dunn, 1998). However their application has only been

experimental although these systems have been observed to occur naturally in sulphate reducing environments. Gilfillan (2000) reported that biofilms most likely consisted of differentiated structures, made up of microbes of different morphologies. Bacteria in biofilms were identified as sulphide oxidizing organisms. Bowker (2000) confirmed the presence of sulphide oxidizing bacteria in biofilms and suggested their possible arrangement within biofilm structures.

Rein (2002) investigated the use of the sulphur biofilms as a system to treat sulphur polluted waters. Molwantwa (2008) reported a detailed explanation on the structural/functional relationship that occurs within biofilms and how this could facilitate the production of elemental sulphur. Van Hille & Mooruth (2011) provided insight into the kinetics and mass balance that occur during the process.

2.4 Sulphide Removal Processes

Sulphide is a toxic, corrosive and odorous compound that should be removed after treatment of acid mine drainage. Various strategies have been used to treat sulphide-rich water. The oxidation of sulphide to elemental sulphur has aroused great interest, given the economic benefits of elemental sulphur in the production of fertilizer and bioleaching processes (Janssen *et al.*, 1999, Rein *et al.*, 2002, Jonhson, 2000, Dvorak *et al.*, 2004; Waterson *et al.*, 2006; Celis-Garcia *et al.*, 2007).

2.4.1 Sulphur Production via the Chemical Pathway

The two significant sulphide oxidation reactions are shown in Equations 2.6 and 2.7 based on studies by Janssen et al., (1999). The reactions show that sulphide can either be oxidized partially to sulphur or completely back to sulphate under different conditions so it was observed that the oxidation of sulphide is crucial in order to avoid oxidation back to sulphate.

$$2HS^- + O_2 \rightarrow 2S^o + 2OH^- \quad (2.6)$$

$$2HS^- + 4O_2 \rightarrow 2SO_4^{2-} + 2H^+ \quad (2.7)$$

In order to achieve partial oxidation of sulphide to elemental sulphur, the stoichiometric ratio of sulphide to oxygen should be kept at 2:1. Other possible products of sulphide oxidation include thiosulphate ($S_2O_3^-$), polythionates ($-SO_3$—Sn-SO_3^-) and polysulphides (Sn^{2-}, n= 2 to 5). Polysulphides have been identified as important intermediates that occur during sulphide oxidation (Yao and Millero, 1996; Steudel, 1996; Janssen *et al.*, 1999; Stuedel, 2000). The oxidative reaction in Equation 2.6 implies that sulphide ions are in contact with oxygen under a narrow pH and Eh range (Also refer to Figure 2.9) conditions to produce elemental sulphur and hydroxide ions. The elemental sulphur produced consists of cyclic S_8 molecules that combine to form larger crystals that can be separated from the solution either by flotation or other physical separation techniques (Steudel, 1996; Janssen *et al.*, 1999; Steudel, 2000).

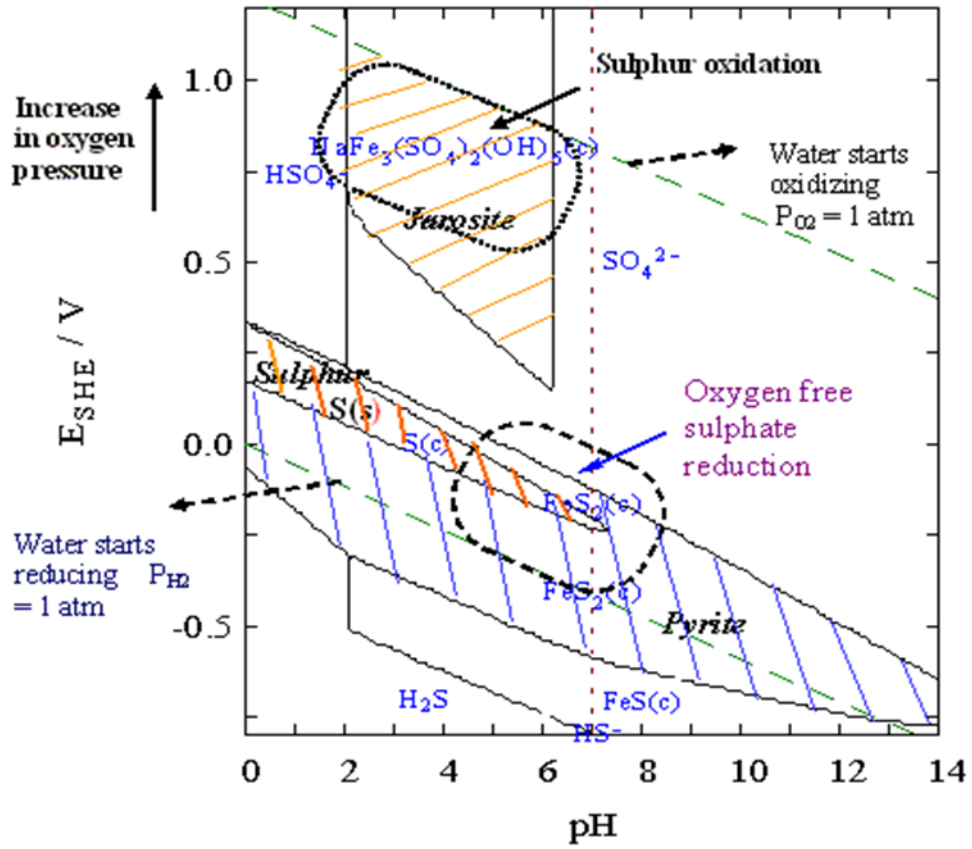

Figure 2.9: Pourbaix Diagram representing stable sulphur compounds in (contact with) aqueous solution at different oxygen pressure (redox potential, *E* in volts) and acidity (*pH*), calculated for the sulphate, iron and sodium ion concentrations in aqueous solution (calculated for $[SO_4^{2-}]_{tot}$ = 350 mM, $[Fe^{3+}]_{tot}$= 50 mM and $[Na^+]_{tot}$ = 400 mM.

2.4.2 Sulphur Production via the Biological Pathway

Biological sulphide oxidation is carried out by sulphide oxidizing bacteria (SOB) which use sulphide as an electron donor and produce sulphur particles in the submicron range (Bruser *et al.*, 2000). The sulphur particles are made of a core of elemental sulphur which is covered by a layer of naturally charged polymers, making the particles hydrophilic (Steudel, 1996; Bruser *et al.*, 2000; Janssen *et al.*, 2000). The known groups of microorganisms involved in the sulphide oxidation include photosynthetic sulphur bacteria, colourless sulphur bacteria and certain heterotrophic bacterial groups.

2.4.2.1 Photosynthetic Sulphur Bacteria

Photosynthetic sulphur bacteria include green and purple sulphur bacteria. These bacteria use sulphide as electron donor, carbon dioxide (CO_2) as carbon source and electron acceptor in the presence of light to produce elemental sulphur (Equation 2.8).

$$CO_2+H_2S \rightarrow CH_2O+H_2O + 2S^0 \quad (2.8)$$

Larsen (1952) explained that under limiting light and CO_2 conditions, sulphur is the major product of sulphide oxidation, whereas sulphate is generated in the presence of abundant light and CO_2.(Equation 2.9):

$$2CO_2+H_2S+2H_2O \rightarrow 2(CH_2O)+H_2SO_4 \quad (2.9)$$

The sulphur produced by these organisms is either intra-cellular (e.g. *Chromatium sp.*) or extra-cellular (e.g. *Chlorobium sp.*) in transitional states or final products, respectively, (Prange and Dahl, 2006). Sulphur K-edge X-ray Absorption Near-Structure Spectroscopy (XANES) is a technique used for sulphur speciation analysis. XANES has been widely used in geochemical (Rowe et al., 2007), soil (Zhao et al., 2006 and Prietzel et al., 2007) and biological samples (Zhao et al., 2007; He et al., 2009). Pickering et al. (2001) reported that extracellular sulphur globules were in the form of S_8, while Prange *et al.* (2002) reported intracellular sulphur globules produced by *Beggiatoa alba* and *Thiomargarita namibiensis* were cyclic, consisting of 8 sulphur atoms and sulphur chains respectively.

Chlorobium limicola is a green sulphur bacterium that has been used for sulphide oxidation in bioreactors, with 90% of sulphide had been converted to sulphur (Johnson, 2000). However, the light requirement complicates the design of the reactor, resulting in high operating costs. Furthermore, the oxidation of sulphide is strictly coupled to bacterial growth, making photosynthetic sulphur bacteria expensive to use for sulphide oxidation (Kim et al., 1990).

2.4.2.2 Colourless Sulphur Bacteria

Colourless sulphide oxidizing bacteria can be found in both archaea and eubacteria domains (Johnson, 2000). During the oxidation processes, oxygen (O_2), nitrate (NO_3), manganese (IV) or iron (III) can be used as terminal electron acceptors. Colourless sulphide oxidizing bacteria exist in diverse species such as *Acidithiobacillus sp., Thiomicrospira sp., Thiospaera sp., Sulfolobus sp., Leptospirillum sp., Acidianus sp., Thermothrix sp., Thiovulum sp., Beggiatoa sp., Thiothrix sp., Thioploca sp., Thiodendron sp., Thiobacterium sp., Macromonas sp., Achromatium sp. and Thiospira sp.* The members of these genera differ in their pH and temperature requirements for growth. In addition, some of the above organisms are capable of denitrification while others are not (Nielsen *et al.*, 2000; Ito *et al.*, 2004). *Thiobacillus* species are the most common studied genera of colourless SOB. They are gram-negative, rod-shaped bacteria that obtain their energy from the oxidation of inorganic sulphur compounds (Lens *et al.*, 2000; Widdel, 1988). Kelly (1985) explained that the wide variety in the genus complicates the identification of the enzymatic pathway which is involved in the sulphur metabolism.

Colourless Sulphur bacteria are either aerobic or anaerobic. Anaerobic bacteria use hydrogen or ferrous iron as electron acceptors. Sublette and Sylvester (1987) observed that *Thiobacillus denitrificans*, characterised as an anaerobe, was able to oxidize sulphide to very low levels under aerobic conditions. Under anaerobic conditions, nitrate is used as the terminal electron acceptor and it is converted to nitrogen (Equation 2.10).

$$5H_2S+8KNO_3 \rightarrow 4K_2SO_4+H_2SO_4+ 4N_2 + 4H_2O \qquad (2.10)$$

Sublette and Sylvester (1987) also observed that *Thiobacillus denitrificans* was responsible for oxidizing sulphide from the gas phase due to its tolerance to high pressure as well as tolerance to other sulphide derivatives such as CS_2, COS and CH_3SCH_3. These compounds are easily partition to the gas phase as are this difficult to treat since in most situations they

will not be available to the bacteria in water. The presence of molecular oxygen increases the tolerance where as its absence reduces tolerance drastically. Cadenhead & Sublette (1990) observed tolerance to sulphur compounds under loadings as high as 15.1 - 20.9 mmol/g/h in the presence of oxygen. The same culture was susceptible to sulphur compounds under loading rates as low as 5.4-7.6 mmol/g/h in the absence of oxygen.

Other species that possess sulphide oxidizing include *Beggiatoa sp.* which has been reported to form a symbiotic relationship with sulphate reducing bacteria under micro-aerophilic conditions, to convert biogenic sulphide to intracellular sulphur or sulphate (Basu *et al.,* 1995).

2.4.2.3 Heterotrophic Sulphur Bacteria

There have not been a lot of studies carried out using heterotrophic bacteria for sulphur oxidation. However, few studies have reported the use of *Pseudomonas putida* to achieve sulphide oxidation (Chung *et al.*, 1996a; Chung *et al.*, 1996b and Huang *et al.*, 1997). The latter indicated that sulphide oxidation in the 5-60 mg/L range can be oxidized in the ratio 15:18:50 (sulphate, sulphite and elemental sulphur, respectively as the major products), with approximately 12% as the residual.

2.5 Factors Affecting Sulphide Oxidation

Buisman *et al.* (1990) reported that organic matter such acetate or glucose had little or no impact on biological sulphide removal, while Brigmon *et al.* (1997) reported that the presence of organic matter stimulated the growth of filamentous sulphide oxidizing bacteria such as *Thiothrix species.* These bacteria are considered undesirable for sulphide oxidation because the generated sulphur is intracellular and the presence of these bacteria may cause serious bulking problems. A previous study done by Rein (2002) demonstrated the importance to develop a sulphur recovery process under heterotrophic conditions. He also reported that the presence of organic matter and heterotrophic bacteria could favour the production of elemental sulphur as the main product. van Hille and Mooruth (2011) then emphasized the crucial role of the carbon source in the sulphide oxidation process. Cultures provided with a simple organic matter as acetate show improved conversion of sulphide to sulphur.

The effect of redox potential on sulphide oxidation has not been widely studied. Janssen *et al.*, (1998) reported a relationship between sulphur production and redox value. The optimal Redox value for sulphur production in a continuous flow gas lift reactor was reported to be between -147 and -137 mV.

2.6 Optimization of Sulphur Recovery

(a) Biotrickling Filter Reactor

Biological oxidation of sulphide to elemental sulphur was observed to be technical feasible when using biotrickling fliter reactors have been observed to remove high concentration of sulphide from gases (Fortuny et al, 2008 and 2011). The trickling liquid velocity (TLV) is important for the attachment of biomass to the packing material for proper gas-liquid mass transfer and for elemental sulphur flushing in case of accumulation. Fortuny et al., (2011) observed that an increased TLV greatly affects the flushing out of accumulated elemental sulphur thus reducing sulphur recovery. Therefore it is still needed to optimize TLV to avoid the accumulation of elemental sulphur on the packing material.

(b) Linear Flow Channel Reactor (LFCR)

The functionality of the LFCR is based on the use of floating sulphur biofilms at the air/water interface. Molwantwa (2008) provided a descriptive model of the different processes that do occur within the biofilm. The reactor consists of 8 parallel channels that operate individually. Each channel is further divided into 8 compartments by a series of under or over baffles which retain the biofilm within each compartment so allowing the harvest of individual compartments, biofilm growth and sulphur production. The reactor operates in such a way that the final compartment is free of biofilm therefore ensuring a complete sulphide oxidation to elemental sulphur. Van Hille & Mooruth (2011) provided an insight into the kinetics and mass balance that occur during the process.

(c) Expanded granular sludge bed (EGSB)

This type of reactor allows the biological treatment of wastewater which passes through at high upflow velocity without the biomass being washed thus accomplishing a high removal rate of organic matter. Chen et al., (2008) were the first to use the reactor to remove simultaneously sulphide, nitrate and organic matter. However Chen et al., (2009) provided a mass balance calculation to justify their findings (Chen et al., 2008) which were higher than anterior studies (Reyes-Avila et al., 2004).

2.7 Chapter Summary

Literature survey shows that chemical and biological methods have been investigated for the treatment of AMD. Biological methods are of great interest compared to chemical treatment procedures. The biological recovery of sulphur has been investigated further by several authors and this has led to development of Linear Flow channel Reactor. In spite of several studies conducted, the optimum conditions for production of elemental sulphur from biological sulphide oxidation remain unknown.

CHAPTER 3

MATERIALS AND METHODS

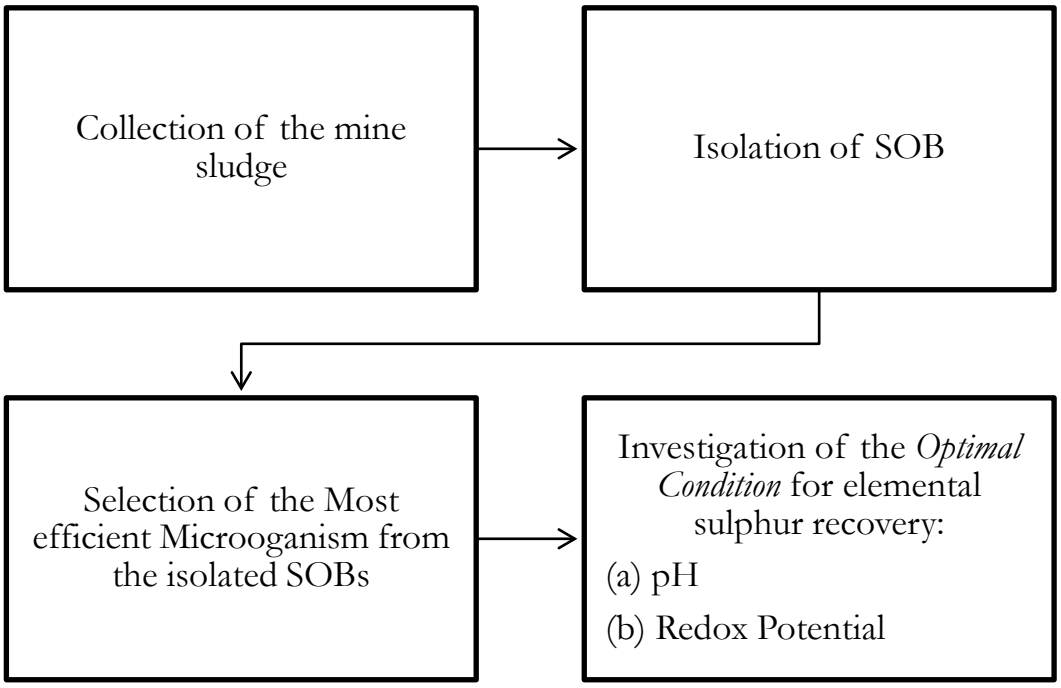

Figure 3.1: Flow Diagram of the various steps taken during the experimental phase

3.1 Chemical Reagents

The chemicals and reagents that were used for successful completion of this study were of analytical grade from Merck, Johannesburg, South Africa.

3.1.1 Preparation of Media and Stock Solutions

All media and stock solutions used throughout the study were autoclaved at 121°C for 15minutes for sterilisation using Hirayama HV 50 autoclave. All chemical reagents used, were accurately weighed using Precisa 4000C balance (Vactech, Johannesburg, South Africa. Sulphide and sulphate stock solutions used in the study were prepared separately as the following: 1.48g of Sodium Sulphate (Na_2SO_4) and 7.50g of Sodium Sulphide ($Na_2S.\ 9H_2O$) were weighted were transferred into 500ml of distilled water then the water was stirred till dissolution of the powders and finally more water was added to make up 1000mL. Both

Solutions were then stored in the freezer at 4°C till usage. 500 mL of sodium lactate was prepared by combining 1M lactic acid and 1M of sodium hydroxide. Both nutrient broth and agar were prepared by dissolving 16g and 23g of nutrient broth and agar respectively into 1000mL of distilled water then the solutions were sterilized for 15minutes at 121°C. After the sterilization process, they were cooled to 50-55°C and nutrient agar was poured into purified petri dishes till solidification. Nutrient glucose and lactate broths were similarly made as the nutrient broth but 5g of glucose and 5g of lactate were added to the broth prior to the disinfection step.

A modified selective medium of Nagarajan et Sudhakar, (2012) for sulphide oxidizing bacteria (labelled (SM_{ox}) was composed of four solutions. Solution A was prepared by dissolving $Na_2S_2O_3$.5H2O (5.0 g), KNO_3 (2.0 g) and NH_4Cl (1.0 g) in 250 mL distilled water. Solution B was prepared by dissolving KH_2PO_4 (2.0 g) in 250 mL distilled water. Solution C was prepared by dissolving $NaHCO_3$ (2.0 g) in 250 mL distilled water. 100 mL solution D was prepared by dissolving $MgSO_4.7H_2O$ (0.8 g) and FeSO4.7H2O (2%w/v) in 100 mL HCl. The solutions A, B, C and D were autoclaved separately at 121°C for 15 minutes and eventually mixed together. The pH was adjusted to 7 using 1N NaOH.

The selective medium for sulphate reducing bacteria (labelled SM_{red}) was prepared by dissolving KCl (0.3 g), $MgSO_4$•$7H_2O$ (3.0 g), $MgCl_2$• $6H_2O$ (2.5 g), NH_4Cl (0.5 g), NaCl (1.0 g), KH_2PO_4 (0.6 g), Sodium lactate (20 L), yeast extract (2.0 g), peptone (2.0), L-cysteine (0.5 g), Ascorbic acid (0.5 g) and $FeSO_4$•$(NH_4)2SO_4$•$6H_2O$ (2.0 g) in 1000 mL distilled water. Ascorbic acid, L-cysteine and $FeSO_4$•$(NH_4)2SO_4$•$6H_2O$ which were separately sterilized with a bacteria filter membrane (0.22 µm of aperture) were added to the medium. The pH was then adjusted to 6.0~6.5, according to the medium preparation procedure by Jiang *et al.*, (2009).

3.2 Microbial Isolation and Enrichment

3.2.1 Microorganisms source

The cells were isolated from dry sludge collected from the final sludge treatment process of a local gold mine in Johannesburg, South Africa. The sludge was poured into sterilized bottles and stored in the freezer at 4°C.

3.2.2 Microbial Isolation

1 g of sludge was inoculated into 10 mL volumetric flask of SM_{ox} prepared. A serial dilution of the inoculated medium was carried out to promote the formation of clear single colonies. 0.1 mL from each diluted sample was then spread onto the plates using the spread technique. Three different types of media were used for the isolation process: Nutrient agar to determine the presence of chemolithotrophic bacteria, nutrient agar plates with glucose and nutrient agar with sodium lactate to check for the presence of chemoorganotrophic bacteria. The different agar plates were incubated at ±30°C in a dark room to avoid the growth of photosynthetic bacteria.

In order to isolate anaerobic, non-photosynthetic sulphate reducing bacteria, 1 g of dried sludge was inoculated into SM_{red}. Oxygen was removed by purging the medium with 99% Nitrogen (N_2) gas for 5-10 min. The purged flasks were then sealed with parafilm. The flasks were kept in a dark room at ±30°C for 3 days to avoid the growth of photosynthetic sulphate reducing bacteria. The medium was observed for colour change. Black coloration of the medium indicates the presence of sulphate reducing bacteria and formation of metallic sulphide precipitates.

3.3 Identification of Sulphide Oxidizing Microorganisms

To identify sulphide oxidizing species, bacteria isolated from dry sludge were grown in the presence of sulphide. The bacteria were characterized by physical morphology first using gram staining technology followed by genotypic identification using the 16S rRNA gene finger printing. Isolated species were evaluated for their sulphide oxidizing capability by observing sulphide removal from solution.

3.3.1 Gram Stain

The gram stain procedure used is the Hucker Method (APHA, 2005). 1 mL of the inoculum of each isolated bacteria grown separately till exponential phase was spread on a microscope slide and heat-fixed. The heat-fixed glass side was immersed in crystal violet and air-dried for one minute. The glass side was gently and directly washed under tap water for a few seconds. Iodine mordant was added and left for one minute. The fixed cells on the glass side were

rinsed again with water for 10 seconds. A safranin solution was added and left for 30 seconds, before rinsing with water again for another 10 seconds. The slides, each containing a different bacterium, were dried with absorbent paper before observation using a ZEISS AXIOSCOP II Microscope equipped with a 100*/1.30 oil plan- NEOFLUAR objective (Carl Zeiss, Oberkochen, Germany). The cells were differentiated by their colour: black-violet and red-pink for gram-positive and negatives cells respectively.

3.3.2 16s rRNA Sequencing

The phylogenetic characterization of isolate species, using conserved regions of their 16s rRNA was carried out. In preparation for the 16S rRNA sequence identification, the isolated colonies were streaked onto nutrient agar followed by incubation at 30°C for 24hours. 6 colonies were obtained. DNA was extracted from the pure cultures derived from the colonies using the DNeasy Tissue kit (QIAGEN Ltd, West Sussex, UK). The 16S rRNA genes were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers pA (corresponding to positions 8-27) and pH1 (corresponding to positions 1541-1522 of the 16S gener) (Coenye *et al.*, 1999). A primer pD corresponding to positions 519-536, was used for internal sequencing. The resulting sequences were deposited in the Genbank of known microorganisms using the basic Blast Tool Search of the National Centre for Biotechnology Information (NCBI, Bethesda, MD). The nucleotide sequences of the 16S rRNA genes were compared with reference sequences from the GenBank database. The 16S rRNA gene sequences of the purified strains were aligned with reference sequences corresponding to sulphate reducing and sulphide oxidizing. Sequence alignment was verified manually using the program BIOEDIT. Pairwise evolutionary distances based on an unambiguous stretch of 1274 bp were computed by using the Jukes and Cantor method (Jukes and Cantor, 1969). Phylogenetic tree diagrams were then constructed using the neighbour-joining method. Confidence in the tree topology was determined analysis based on 100 re-sampling.

3.3.3 Sulphide Removal Test

Each isolated cultures at their exponential phase were used in order to conduct the sulphide oxidation tests in 250 mL Erlenmeyer flasks in which 100 mL of sterilized nutrient broth was

amended with sulphide to give a final concentration of 20-60 mg/L. 2 sets of tests were conducted: the first set being the control and the second test a heterotrophic condition. In this study lactate and glucose were tested separately to determine which of these carbon source facilitated higher sulphide oxidation.

3.3.4 Total Biomass Analysis

5 mL of bacteria culture was taken from each flask every 6 hours and centrifuged for 10 minutes at 6000 rpm. The pellet obtained was used for total biomass analysis. The pellet was re-suspended in 1mL distilled water and filtered through a pre-weighed Whatman filter paper. The filter paper was dried in the oven at 75-80°C to obtain a dry weight value. The difference between the dried filter paper with cells and the empty filter paper was considered to be the total dry cell biomass.

3.3.5 Viable Biomass Analysis

1 mL of bacteria culture was taken from each flask every 6 hours. Samples were serially diluted into 9 mL sterile 0.85% NaCl solution. 0.1 mL of the diluted samples was transferred into plate count agar using the spread plate technique. The plates were incubated for 24 hours at ±30°C. The colonies were counted after incubation and multiplied by the dilution factor. The bacterial count was reported as colony forming units (CFU) per mL of sample.

3.4 Batch Experiments

3.4.1 Effects of pH and Redox Potential on Sulphide Oxidation

These experiments were designed to identify the effect of pH and redox potential on sulphide oxidation. This phase of the study was further split into 3 experimental phases (EP). During EP1, isolated bacteria were left to grow and adapt themselves to the pH and redox conditions at ±30°C and served as the control experiments. During EP 2, the effect of pH on bacteria growth was monitored while EP3 involved the investigation of redox potential.

The Redox potential and pH values were measured using a WTW pH/mV 330 meter (Merck, Johannesburg, South Africa). Each test was conducted as duplicate sets.

Table 3.1: Experimental Plan for Investigating Effects of pH and Redox Potential on Sulphide Oxidation

EP	Time	pH value	Eh	Temperature
EP 1	7 days	7	Not controlled	30°C
EP 2	4 hours	6-8	Not Controlled	30°C
EP 3	4 hours	6-8	-130 mV-80mV	30°C

3.4.2 Sulphate Batch Tests

The aim of the test was to investigate the influence of different feed of Chemical Oxygen Demand (COD)/sulphate ratio on the sulphate reduction rate. The test was conducted in 500 mL Erlenmeyer flasks numbered from R1 to R5. 50 mL sulphate reducing bacteria was inoculated in a 200 ml mixture in 1:1 ratio (v/v), which consists of SM_{red} and synthetic acid mine drainage, prepared by adding Mg $SO_4.7H_2O$ (10 g), $Fe_2(SO_4)_3.XH_2O$ (20 g), $ZnSO_4$ (5 g) and $CuSO_4$ (0.5g) in 1000 mL tap water. The total sulphate concentration was determined to be 650 mg/L and was maintained constant throughout the whole experiment but the sodium lactate concentration was different in the medium 435.5; 1105; 1462.5; 1950 and 3250 mg/L using feed ratios COD/SO_4^{2-} of 0.67; 1.7; 2.25; 3 and 5 respectively (see Table 3.2). Oxygen was removed from the medium by purging the batches with 99% Nitrogen (N_2) gas for 10 minutes before the flasks were sealed with foil and parafilm. The samples were incubated at ±30°C. The sulphate reduction phase was monitored by taking samples every 12 hours by means of plastic syringes. The samples were then centrifuged in 2 mL eppendorf tubes at 6000 rpm for 10 min using a Minispin® Micro-centrifuge (Eppendorf, Hamburg, Germany). The tests were conducted as duplicate sets.

Table 3.2: COD/Sulphate Ratio

Sample Nr	R1	R2	R3	R4	R5
[SO_4^{2-}] (mg/L)			650mg/L		
COD/SO_4^{2-} ratio	0.67	1.7	2.25	3	5

3.5 Mixed Batch Reactor System

A batch reactor with a total volume of 2L consisted of a glass wall vessel equipped with gas samples, pH and ORP probes and gas purging lines (Figure 3.1). At the beginning of the sulphate reducing phase, nitrogen gas was added to decrease the redox potential in order to promote sulphate reducing conditions. During the second phase of the experiment, a suitable bacteria culture was added to facilitate the oxidation of sulphide.

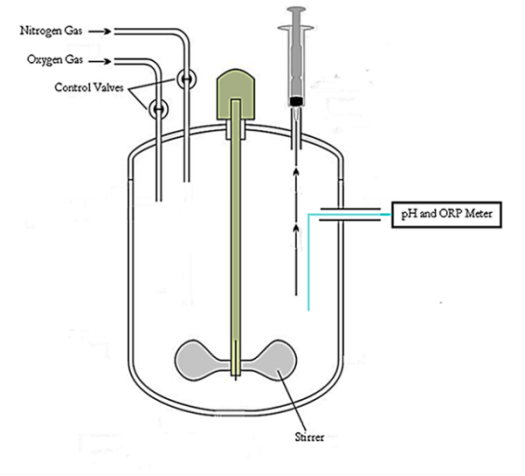

Figure 3.2: Schematic diagram of the reactor used for elemental sulphur production during the complete treatment of synthetic acid mine drainage.

3.6 Analytical Methods

3.6.1 ICP-OES Analysis

The concentration of elemental sulphur (S^0) in solution was determined using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Varian Vista Pro; CCD Simultaneous, Australia). Samples obtained were diluted and filtered to an ICP tube using a disposable syringe (10 ml NORM-JECT, Latex free) through a syringe filter (0.2 µm Supor membrane from Pall Corporation, U.S.A.). All analyses were carried out in triplicate. 90% of the samples were under detection limit of sulphur.

3.6.2 UV/Vis Spectrophotometry

UV/Vis spectrophotometry was used to determine the concentrations of sulphide (S^{2-}), sulphate (SO_4^{2-}).

Sulphide: The concentration of sulphide was determined using a Spectroquant Sulphide Test Kit (Merck, Johannesburg South Africa). The bottles in the kit were labelled S^{-1}, S^{-2} and S^{-3} respectively. 1 mL of bacterial supernatant was added to 9 mL distilled water in a 10mL volumetric flask. 5 mL of each diluted bacteria sample was transferred into different test tubes. 1 drop of S^{-1} was added to each test tube and mixed for 15-30 seconds. 5 drops of S^{-2} was added to each test tube and mixed for 15-30 seconds. 5 drops of S^{-3} was finally added to each test tube and mixed for 15-30 seconds. The mixture was then let to stand for about a two minutes for full colour development. The colour formed was measured at a wavelength of 665 nm using a UV spectrophotometer (WPA, Light Wave II and Labotec, South Africa).

Sulphate: 1 mL of sample was added to 99 mL with distilled water in a 250 mL Erlenmeyer flask. 5 mL of conditioning reagent was added to the flask, the solution was mixed by means of a magnetic stirrer and (0.2-0.3 g) of Barium Chloride ($BaCl_2$) crystals was added to the solution during stirring. The solution was let to stir for one minute at constant speed. Immediately after the stirring period, the mixture was poured into an absorbance cell for turbidity measurement at wavelength of 420 nm, using a UV spectrophotometer. Readings for a sample were recorded over a 4 min period after analysing 30 seconds between readings. The final reading was recorded after the readings became stable.

3.6.3 Mass Balance Analysis

Mass balance analysis was performed in order to determine the most suitable environmental condition for sulphur formation. Mass balance was calculated to account for the initial and final sulphide (S^{2-}), sulphate (SO_4^{2-}) and elemental sulphur (S^0).

$$\text{Mass}_{\text{Accumulated}} = \text{Mass}_{\text{Input}} - \text{Mass}_{\text{Output}} + (\text{Mass}_{\text{Generated/ Destroyed}}) \quad (3.1)$$

In a batch reactor there is no input or output therefore the above equation is transformed to:

$$\text{Mass}_{\text{Accumulated}} = + \text{Mass}_{\text{Generated/ Destroyed}} \quad (3.2)$$

$$\text{Mass}_{\text{Accumulated}} = V\,(dC/dt) \text{ and Mass}_{\text{Generated/Destroyed}} = V\,(dC/dt)_{\text{reaction only}} \quad (3.3)$$

$$(dC/dt) = (dC/dt)_{\text{reaction only}} \quad (3.4)$$

$$\text{Sulphide Removal Efficiency} = \{([\text{Sulphide}]_{\text{initial}} - [\text{Sulphide}]_{\text{final}}) \div [\text{Sulphide}]_{\text{initial}}\} * 100$$

$$\text{Sulphur recovery (\%)} = ([\text{Sulphur}]_{\text{final}} \div [\text{Sulphur}]_{\text{initial}}) * 100$$

3.6.4 Microscopic Analysis of Isolated Bacteria

Microscopy was used to visualise extracellular and intracellular sulphur globules and sulphur deposition on cell surfaces. Surface scanning techniques (FPX) were used to evaluate elemental species distribution inside and outside the cells.

3.6.4.1 Scanning Electron Microscopy (SEM)

SEM was used to visualise the presence of extracellular sulphur globules. Agar gel plates containing microbial colonies were first excised and trimmed to small sizes. The gel blocks were fixed in 2.5% glutaraldehyde for of 15 min. After the fixation step, the cells were rinsed with phosphate buffer to remove any excess fixative. The cells were rinsed once for 10 min then three times for 20 min. The cells were then dehydrated by immersing in graded series of ethanol. They were immersed in 50% ethanol for 5 min, 70% ethanol for 10 min, 80% ethanol for 10 min, 90% ethanol for 15 min and finally twice in 100% ethanol for 20 min. The cells were then dried using the critical point drying method before mounting on metallic stubs using a double sticky tape. The cells were then coated with gold a conductive metal to increase their conductivity in the microscope and to avoid the build-up of high voltage charges on the cells.

3.6.4.2 Transmission Electron Microscopy (TEM)

TEM was also used to reveal the presence of intracellular sulphur globules since it allows the cells to be viewed at very high magnifications compared to SEM. The cells were cleaned to remove unwanted deposits. Phosphate buffer was used to rinse the cells three times for 10

min at room temperature. The cells were prefixed in 2.5% glutaraldehyde solution. Excess glutaraldehyde solution was removed by rinsing the cells with phosphate buffer once for 10 min then three times for 20 min. The secondary fixation was performed to preserve the structure of the cells and to protect the cells: the cells were fixed with 1% osmium tetroxide for 1.5 hours at room temperature. This was followed by dehydration in graded series of ethanol: 50% ethanol for 5 min, 70% ethanol for 10 min, 80% ethanol for 10 min, 90% ethanol for 15 min and 100% ethanol twice for 20 min at room temperature. The cells were then immersed in propylene oxide twice for 20 min at room temperature because ethanol is not miscible with the plastic embedding; the process was used for alcohol substitution. This was followed by the immersion of the cells in resin at room temperature overnight in a fume hood to allow evaporation of resin. The next day the cells were immersed in pure resin for 2 hours at room temperature. Polymerization of the epoxy mixture was achieved by placing the cells in a drying cabinet for 2 days at 40°C and for additional 2 days at 60°C. The cells were then cut into ultrathin slices and stained with uranyl acetate followed by lead citrate. The sections were then mounted in immersion oil.

CHAPTER 4

RESULTS

4.1 Removal of sulphide under heterotrophic conditions

From the dry sludge, 6 distinct microbial species of which bacteria and filamentous microorganisms were isolated. The bacterial population showed the presence of both gram positive and negative species (See Figure 1, Appendix A) which were in agreement with Gilfillan (2000), Bowker (2000) and Molwantwa (2008) findings.

The 16S rRNA sequencing resulted in the identifications of the possible phenotypes with 99% probability match as shown in Table 3.1 and the phylogenetic trees of the bacterial species were shown in Figure 2 (Appendix A).

Table 4.1: List of the sulphide oxidizing microorganisms isolated from dry gold mine sludge

Sample Name	Blast Results	Max ID (%)
X1	*Micrococcus luteus*	99%
X2	*Lysinicibacillus fusiformis*	99%
X3	*Lysinicibacillus sphaericus*	99%
X4	*Pseudomona putida*	99%
X5	*Fusarium oxysporum*	98%
X6	*Penecillium simplicissimum*	98%

The Sulphide Removal Test was aimed at selecting the most efficient microorganisms for sulphide removal and also to investigate the effect of the carbon sources: lactate and glucose on sulphide removal. The carbon sources were both added at a concentration of 5 g/L for each experiment. Only organisms with sulphide removal rates above 90% were selected as the most efficient sulphide oxidizing microorganisms for later use.

4.2.1 Rate of removal at 50 mg/L Sulphide

Figure 4.1 illustrates that each microbial species reacted differently depending on the carbon source present. The species X_1, X_5 and X_6 (identified in Table 4.1) achieved complete removal of sulphide in the presence of lactate 4 hours after incubation, whereas species X_2 only achieved complete removal of sulphide in the presence of glucose. Contrarily species X_4 did not show any difference in sulphide removal rate in the presence of either carbon sources. Based on the observed results, the strains were classified into two different groups, i.e., the lactate group and the glucose group. The lactate utilizing group consisted of X_1, X_5 and X_6 while the glucose group of X_2, X_3 and X_4.

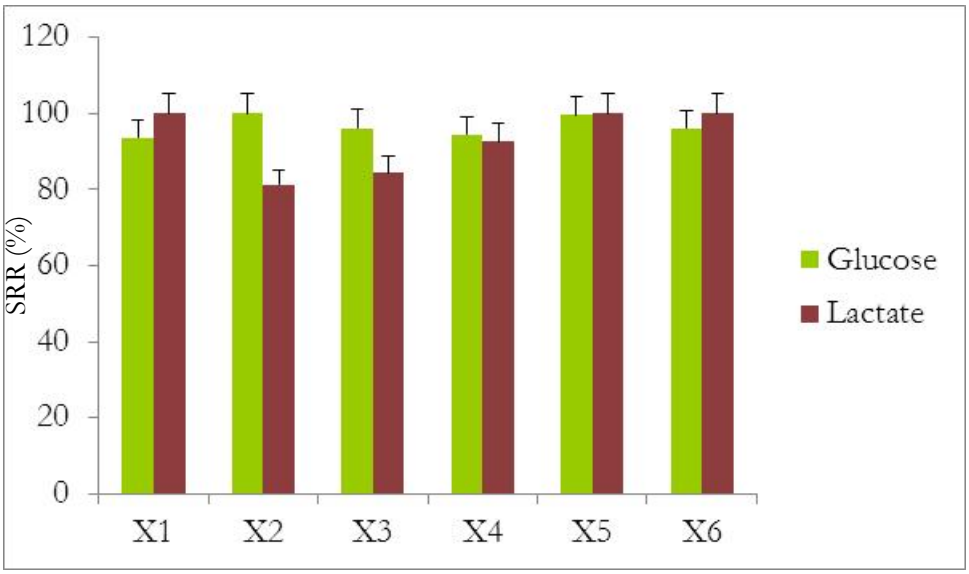

Figure 4.1: Sulphide removal efficiency by individual species under lactate and glucose as solo added carbon sources

4.2.2 Rate removal at 57 mg/L Sulphide

a) Lactate Group

Based on Figure 4.1, species X_1, X_5 and X_6 were selected for a further study. The other species X_2 and X_3, although they oxidized sulphide successfully, their oxidation rates were 15% lower than the oxidized sulphide in the presence of glucose as the solo carbon source. Using species X_1, X_5 and X_6, a concentration-time series batch experiment was conducted and the results on sulphide removal are shown in Figure 4.2.

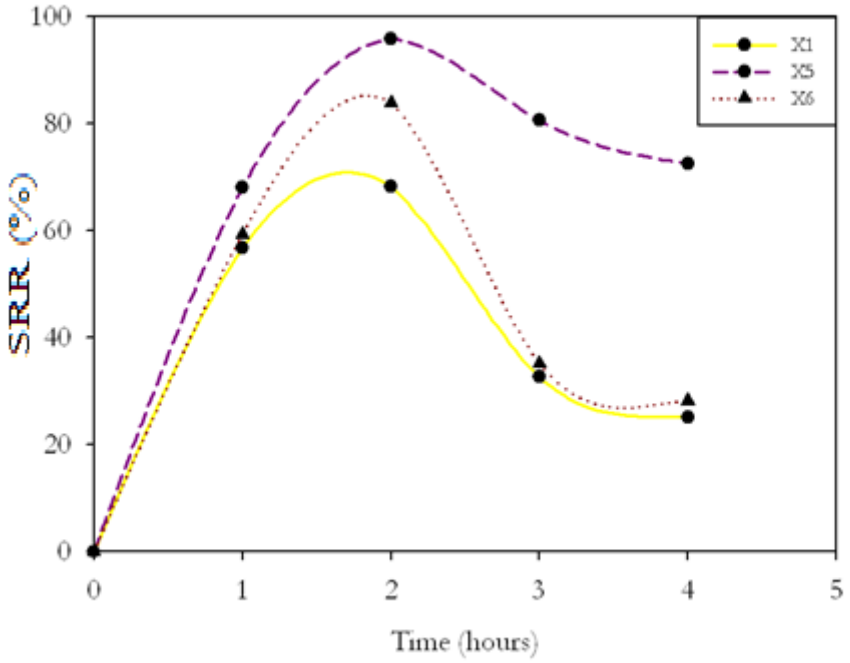

Figure 4.2: Sulphide removal efficiency in the presence of lactate

It was observed that the species X_5 and X_6 achieved the best oxidation rate with X_5 having the highest sulphide removal rate (95.8%) and X_1 being the least efficient with a sulphide removal efficiency of 68.25%. Sulphide concentration increased in each flask with more than 50% increase for X_1 and X_6. The increase in sulphide concentration was assumed to be due to the active sulphate reduction taking place as a result of the readily available lactate present in the reactor. The presence of lactate was said to stimulate the growth of sulphate reducing bacteria so increasing the microbial diversity and finally making the sulphide oxidation less efficient (Kaksonen, 2004; Oyekola, 2008).

b) Glucose Group

From the cultures grown on glucose, the species X_5, X_2 and X_3 strains showed the best results (Figure 4.1). However, X_2 and X_3 were observed earlier to also be able to grow in the absence of organic matter. Therefore they were tested for sulphide removal in the presence and absence of a carbon source. Figure 4.3 illustrates that sulphide removal by *Lysinicibacillus sp.* with glucose as the carbon source, was similar to sulphide removal in the absence of any carbon source after incubation for 4hours (up to 95% of sulphide removal efficiency was

recorded). The lowest sulphide removal rate was recorded when lactate was supplied as the carbon source.

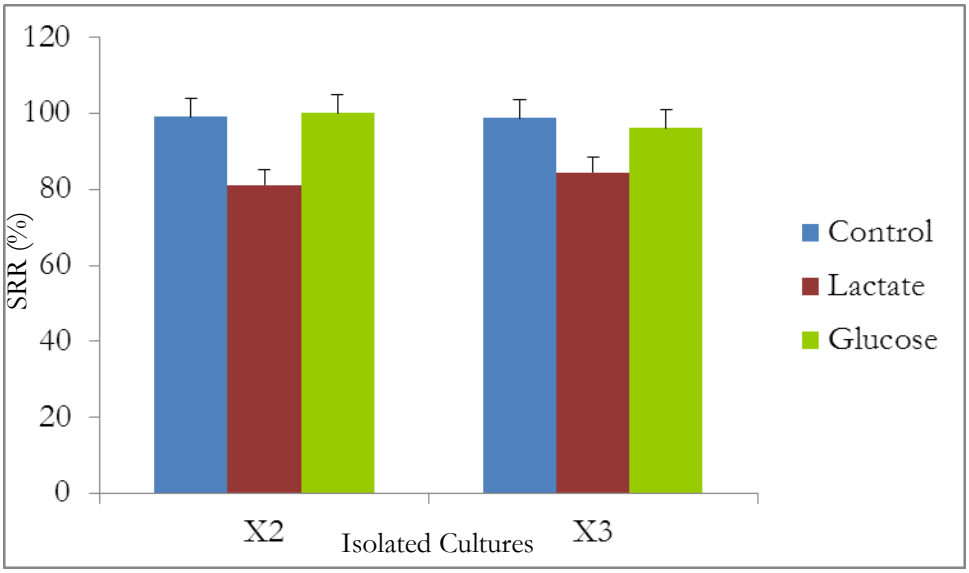

Figure 4.3: Sulphide Removal rate by *L. fusiformis* and *L. sphaericus* after 4 hours of incubation

The ability of the *Lysinicibacillus sp.* to remove sulphide with or without glucose was recognized as a beneficial attribute for sulphide removal under heterotrophic conditions. Figure 4.4 shows sulphide removal rate by individual and mixed cultures when the initial sulphide concentration was 57 mg/L.

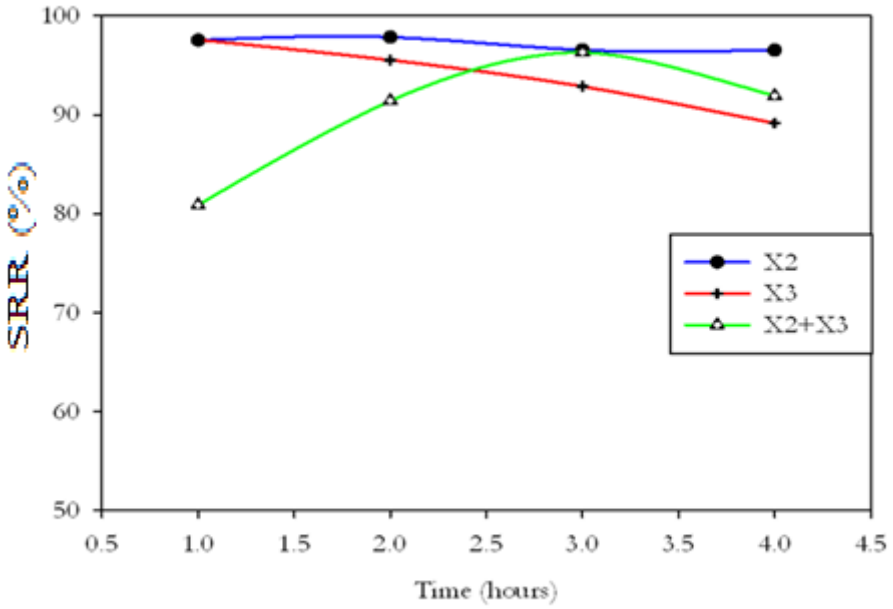

Figure 4.4: Sulphide removal rate in the presence of glucose

Lysinicibacillus fusiformis showed constant sulphide removal efficiency throughout the test and had the highest efficiency (97.84%) while *Lysinicibacillus sphaericus* was decreasing as the incubation time was progressing. However, the mixed culture (X_2 + X_3) showed the lowest removal efficiency at the beginning of the test but as the time passed, the mixed strains reached its maximal sulphide oxidizing rate (96%) before sulphide concentration increased. The sulphide concentration was recorded to increase by 4.57%; 4.00% and 1.34% in (X_2 + X_3), X_3 and X_2 flasks respectively and it was assumed to be due to sulphate reduction. The reported performances of both *L. fusiformis* and the mixture of both *Lysinicibacillus sp.* were similar to the sulphide removal efficiency reported by Chen et al., (2009). For this reason they were chosen for further studies and to also compare between single and mixed species.

4.3 Investigation of Optimal Conditions

4.3.1 Optimum pH Values

The pH of a solution is known to affect both bacterial activity and the type of sulphide species present. The effect of pH on sulphide oxidation was investigated using various pH

values ranging between 6 and 8 with the same sulphide concentration in the reactor. This range was chosen based on a report by Donfeng *et al.,* (2011), stating that the range of pH (6 to 8) was the optimal pH range for the bacterial activity and the pH of the sulphide rich effluent from an acid mine drainage treatment always fall within the same range (Cao et al., 2009 and Singh et al., 2011).

4.3.1.1 Effect of pH on *Lysinicibacillus fusiformis* on sulphide removal activity

The results of the tests are illustrated in Figures 4.5 and 4.6. Figure 4.5 illustrates the sulphide removal rate over time at different pH values, while Figure 4.6 shows the average sulphide removal rate at the end of the experiment. In Figure 4.5, it was observed that sulphide removal rate was above 90% at every pH value. The sulphide concentration fluctuated in each flask: at pH 6 the concentration was constantly changing, while at pH 7 the concentration remained constant, only after 80 minutes with an average of percentage decrease of 2%. At pH 8, the sulphide concentration only decreased of 1.5% at 100 minutes, this was considered not to be significant.

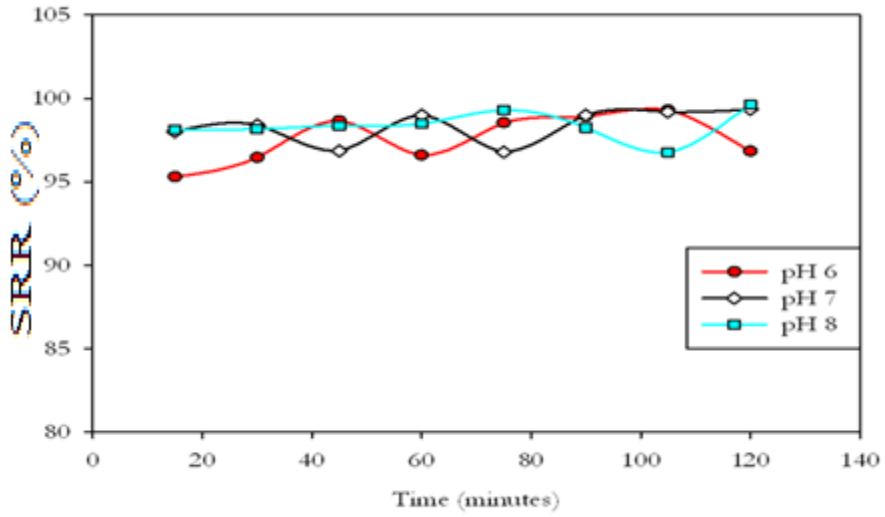

Figure 4.5: Sulphide Removal Rate at the different pH values**.**

At pH 6, H_2S is the dominant sulphide species but as the pH increases towards 8, bisulphide (S^{2-}) ions tend to be dominant in the solution. S^{2-} ions are soluble therefore they are considered to be undesirable species that should be removed. Mamashela (2002) reported

that the H_2S species are not readily oxidized so the rate of sulphide oxidation tends to be lower. This is confirmed by the results obtained which show sulphide concentration variation throughout the experiment (Figure 4.5) and the lowest sulphide removal efficiency (Figure 4.6). As the pH increased, the oxidation rate increased with the highest sulphide removal efficiency recorded at pH 8 which was on agreement with findings reported by Chen et al., (2008) and Xu et al., (2012).

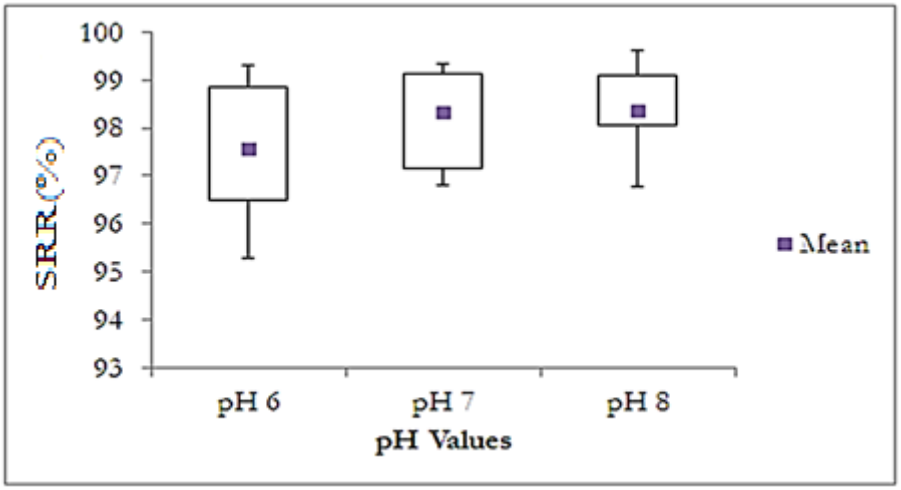

Figure 4.6: Box and Whisker plots showing the mean distribution of the sulphide removal rate by *L. fusiformis*

4.3.1.2 Effect of pH on Sulphide Removal Activity by Mixed Culture

At pH 8, the sulphide removal occurred slowly (Figures 4.7 and 4.8). While sulphide removal efficiencies were at its highest at pH values of 6.7 and 7. Mamashela (2002) suggested that S^{2-} ion species remained in the solution, therefore they were not oxidized. It was also assumed that a competition between the *Lysinicibacillus species* over the substrate may have also affected the ability of the mixed bacteria to oxidize sulphide at pH 8. Figure 4.7 illustrates the change of sulphide concentration over time, while Figure 4.8 shows the sulphide removal rate at the end of the experimental test.

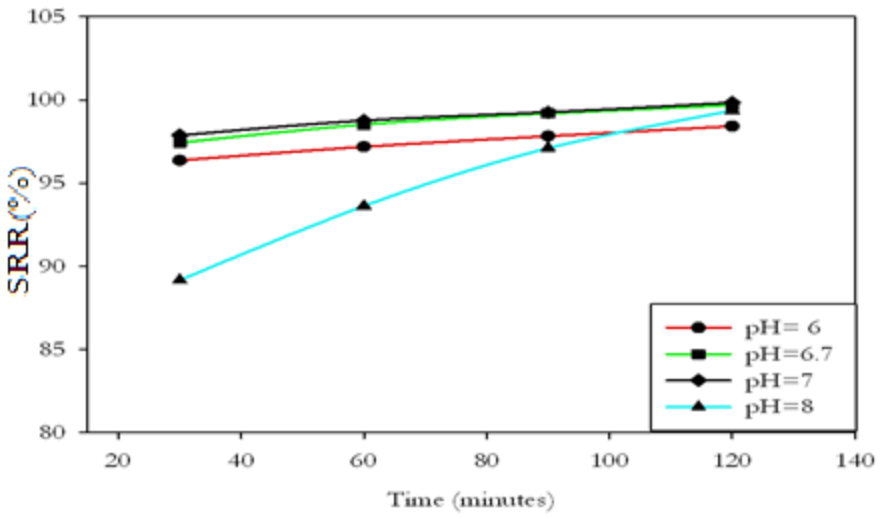

Figure 4.7: Sulphide Concentration at different pH values

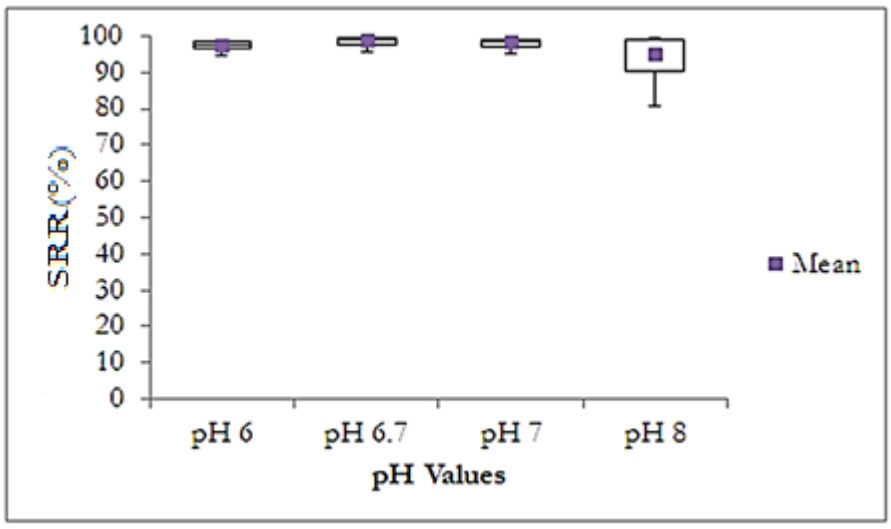

Figure 4.8: Box and Whisker plots showing the mean distribution of the Sulphide removal rate by both *L. fusiformis* and *L. sphaericus*

4.3.2 Oxidation Reduction Potential (Eh)

The aim of the experimental test was to determine the optimum conditions in term of Redox potential for partial sulphide oxidation

4.3.2.1 Effect of Eh on sulphide removal activity by Mixed Culture

Steudel (1996) reported that at a redox potential (Eh) of -130 mV elemental sulphur was generated. For this reason, the mixed strains were tested to confirm elemental sulphur generation under this redox potential but at pH values of 6.7 and 7. Eh was increased from ≃-210 mV to -130 mV by adding oxygen gas into the reactor. Figure 4.9 shows the change in sulphide concentration, over time at different pH and Eh values. It was observed that as the Eh values increased the sulphide removal rate increased further which was on agreement with Molwantwa (2008). The straight and dotted lines in Figures 4.13 and 4.14 represent the original and modified Eh respectively.

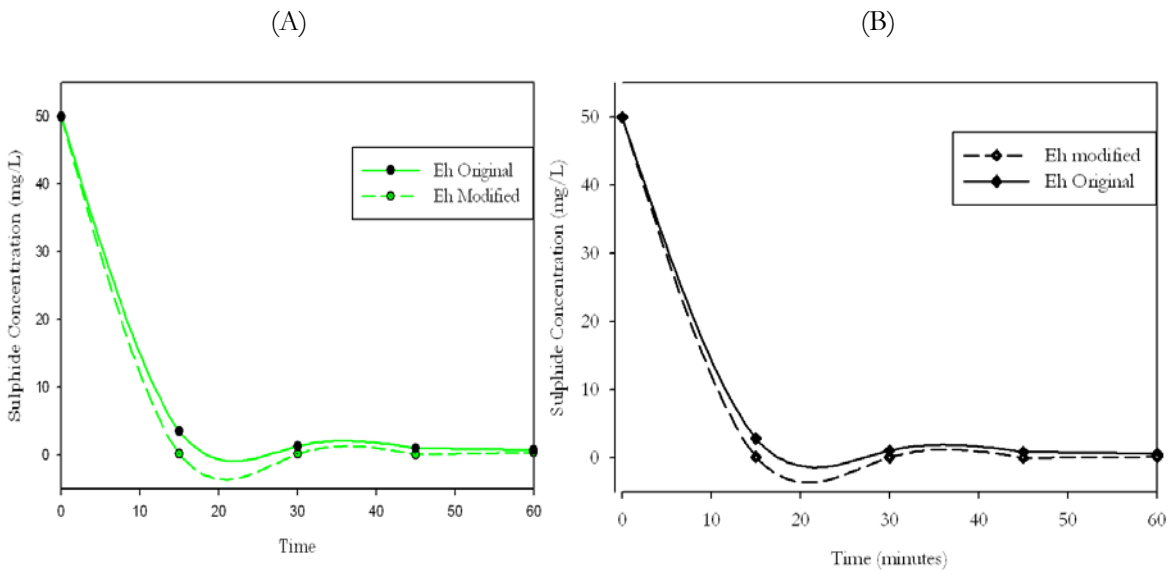

Figure 4.9: Sulphide concentration over time using mixed culture at (A) pH 6.7 and (B) pH 7

4.3.2.2 Fate of Elemental Sulphur in the Reactor.

The quantitative determination of the elemental sulphur generated was done by the mass balance calculation as explained by Chen et al (2009):

$$[S_{in}^{2-}] + [SO_4^{2-}{}_{in}] + [S_2O_{3in}^{2-}] + [S_{out}^{2-}] + [SO_4^{2-}{}_{out}] + [S_2O_{3out}^{2-}] = [\text{production of (biomass-S} + S^0] \quad (4.1)$$

Since a batch reactor was used in this study no input and output were catered for. Initial and final concentrations were measured and thiosulfate ($S_2O_3^{2-}$) concentration was not measured because the biological activity was low (van den Bosh *et al.*, 2008), therefore (4.1) was modified to:

$[S_{initial}^{2-}] + [SO_4^{2-}{}_{initial}] + [S_{final}^{2-}] + [SO_4^{2-}{}_{final}] = [\text{production of (biomass-S} + S^0]$ (4.2)

The results are presented in Tables 4.2 and 4.3:

Table 4.2: Reactor performance on sulphur containing compounds in the batch reactor during the experimental phase (EP 3) at pH 6.7

Time (minutes)		**Sulphide (mg/L)**		**Sulphate (mg/L)**		**S^{2-} removal rate (%)**	**S^0 production (mg/L)**	**S^0 recovery (%)**
Initial	final	$S_{initial}^{2-}$	S_{final}^{2-}	SO_4^{2-} initial	SO_4^{2-} final			
0	15	50	0.11	0	4.62	99.78	45.27	90.54
15	30	0.11	0.11	4.62	17.34	0	-12.72	-
30	45	0.11	0.009	17.34	19.64	91.82	-2.19	-
45	60	0.009	0.37	19.64	25.42	-	-.6.14	-

Table 4.3: Reactor performance on sulphur containing compounds in the batch reactor during the experimental phase (EP 3) at pH 7

Time (minutes)		**S^{2-} (mg/L)**		**SO_4^{2-} (mg/L)**		**S^{2-} removal rate (%)**	**S^0 production (mg/L)**	**S^0 recovery (%)**
Initial	final	$S_{initial}^{2-}$	S_{final}^{2-}	SO_4^{2-} initial	SO_4^{2-} final			
0	15	50	0.17	0	9.24	99.66	40.59	81.18
15	30	0.17	0.07	9.24	18.5	58.82	-9.16	-
30	45	0.07	0.04	18.5	26.58	42.86	-8.05	-
45	60	0.04	0.2	26.58	28.9	-	-2.48	-

From Tables 4.2 and 4.3, it can be deduced that 10 minutes after inception of the test, 99% of sulphide was removed in both reactors. Thereafter, a decrease in sulphide removal was noted with an average of 64% and 67% at pH 6.7 and 7. This confirmed previous

observation: as the pH increase, the rate also increases (Mamashela, 2002). At the end of the test, the sulphide concentration was observed to increase to over 100%.

In both reactors, maximal sulphur concentration was observed to occur 10 minutes within the beginning of the experiment afterwards, it gradually decreased while the sulphate concentration was observed to gradually increase. This could be explained by the oxidation of sulphur to sulphate after 10 min and the negative values of sulphur in Tables 4.2 and 4.3.

Elemental sulphur formation was observed to be higher at pH 6.7 with a sulphur recovery of 90%. For this reason, any batches were subsequently incubated at pH 6.7 to observe the presence of sulphur deposits. An example of sulphur deposition of cells at pH 6.7 is shown in the Figure 4.10 (below).

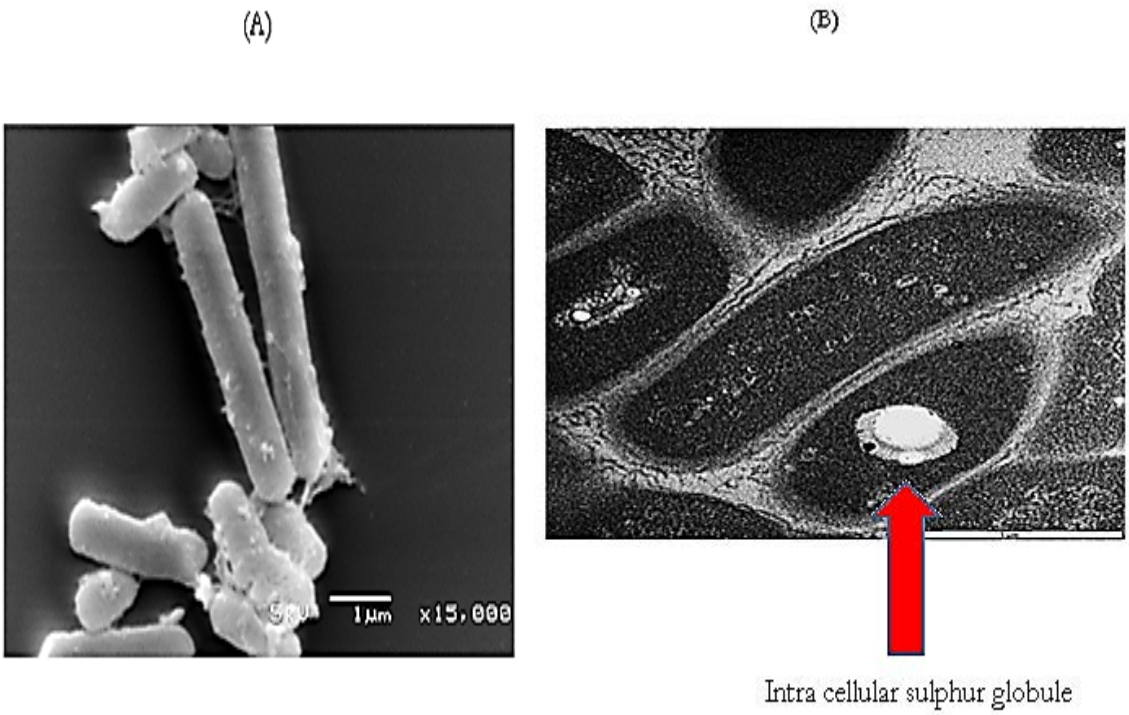

Figure 4.10: (A) Scanning electron micrographs and (B) Transmission electron micrographs of elemental sulphur

In Figure 4.10 (a) extracellular sulphur globules were observed with the scanning electron microscope. The Transmission electron micrograph (Figure 4.10 (b)) showed an inclusion

that was observed to be an intra-cellular sulphur globule. INCA analysis was used to confirm the presence of sulphur within the cells. Low sulphur concentration was observed due to the use of ethanol which removes sulphur during the preparation of the cells for the SEM and TEM (Bruser et al., 2009).

4.3.2.3 Molar Mass Balance on Sulphur species

This exercise was conducted because mass can only be conserved. All sulphur in the system must then be accounted for. Figure 4.11 illustrates the cumulative mass balance among the sulphur-containing compounds at pH 6.7 and Eh 130 mV.

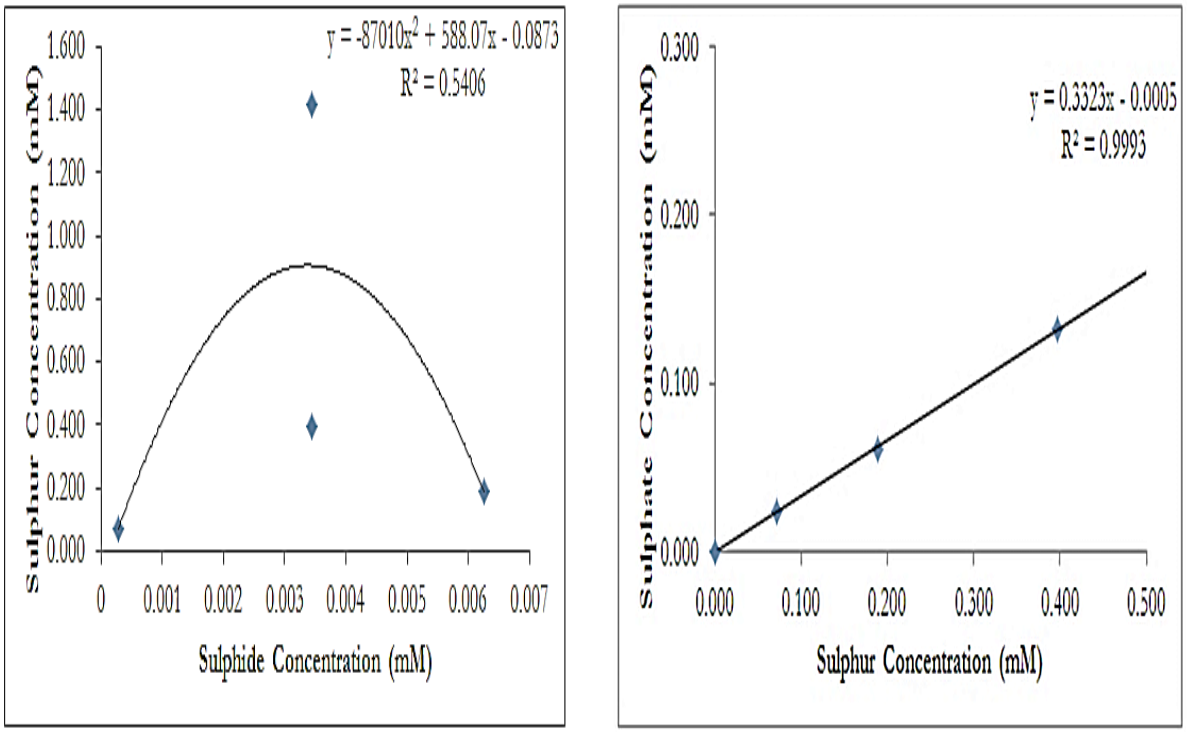

Figure 4.11: Mass balance analysis of the sulphur containing compound at pH 6.7 and Eh=-130mV.

From the above Figure (Figure 4.11 A), it was observed that the oxidative reaction of sulphide to elemental sulphur respect a polynomial curve at the second degree. The results obtained were on agreement with earlier studies (Liu et al., 2004, Romano, 2012 and Nwoye et al., 2013) which concluded that the effect of oxygen on the sulphide oxidation rate to

elemental sulphur was not a linear trend but a polynomial one. This also explained that several other reactions may have taken place in the solution just like Romano (2012) stated.

During the biooxidation of sulphide, Thurston et al (2010) proposed the oxidation of sulphide to sulphate occurs via an intermediate sulphur species which was then found to be elemental sulphur by Heidel and Tichomirowa (2011). A linear regression line fits the model proposed by Heidel and Tichomirowa (2011) stipulating the oxidation of sulphate via sulphur. Heidel and Tichomirowa findings (2011) were in accordance with above results so confirming that sulphur oxidation to sulphate fits a linear trend therefore describing the process to be stable and confirming that every moles of sulphur were directly converted to moles of sulphate.

4.3.2.4 Effect of Eh on sulphide Removal Activity by *Lysinicibacillus fusiformis*

It was observed earlier that the pH values do not intensely affect the rate of sulphide removal by *Lysinicibacillus fusiformis,* however the best removal rates were observed at pH 8. It was also observed from the studies carried out with the mixed culture that at Eh -130 mV, sulphur was produced confirming results presented by Steudel, 1996. On the other hand, Molwantwa, (2008) observed that Eh could get around -100 mV for sulphur generation. Figure 4.12 shows the changes in sulphide concentration over time at pH 8 and Eh -80 mV. It can be observed that as the Eh increases, the sulphide oxidation rate also increases.

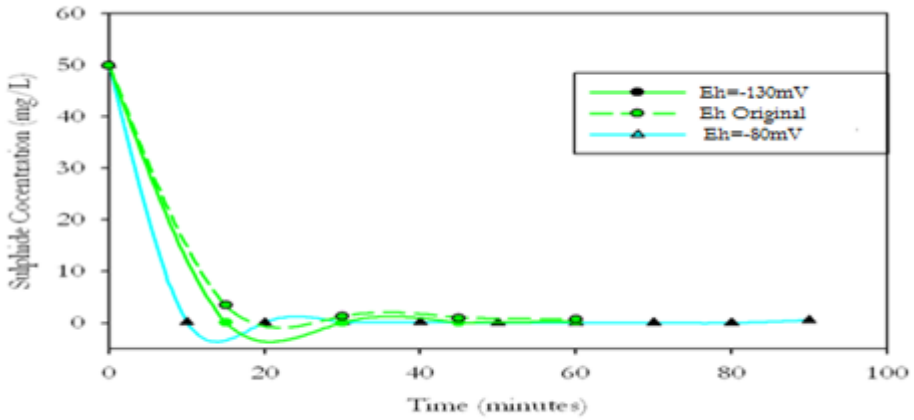

Figure 4.12: Sulphide concentrations at pH 8 and Eh -80mV

Table 4.4 shows the mass balance analysis for sulphide removal under the conditions of pH 8 and Eh = -80mV. Figure 4.13 illustrates the cumulative mass balance among the sulphur containing compounds under these conditions.

Table 4.4- Reactor performance on sulphur containing compounds in the batch reactor at pH 8 and Eh=-80mV

Time (minutes)		**S^{2-}(mg/L)**		**SO_4^{2-} (mg/L)**		**S^{2-} removal rate (%)**	**S^0 production (mg/L)**	**S^0 recovery (%)**
initial	final	$S_{initial}^{2-}$	S_{final}^{2-}	SO_4^{2-} initial	SO_4^{2-} final			
0	10	50	0.153	0	2.3	99.69	47.55	95%
10	20	0.153	0.096	2.3	3.59	37.25	-1.23	-
20	30	0.096	0.11	3.59	5.08	-	-1.50	-
30	40	0.11	0.159	5.08	6.66	-	-1.63	-
40	50	0.159	0.079	6.66	8.11	50.31	-1.37	-
50	60	0.079	0.062	8.11	9.26	21.52	-1.13	-
60	70	0.062	0.057	9.26	13.21	8.06	-4	-
70	80	0.057	0.043	13.21	15.33	2.46	-2.11	-
80	90	0.43	0.57	15.33	20.31	-	-5.12	-

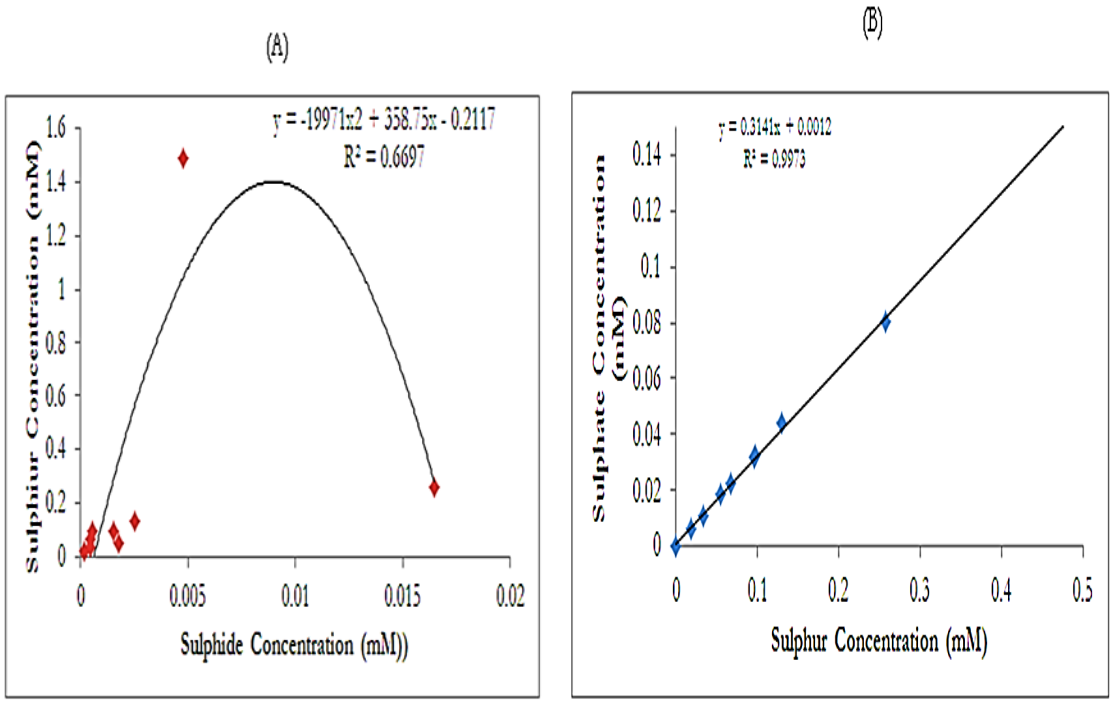

Figure 4.13: Mass balance analysis of the sulphur containing compound at pH 8 and Eh=-80 mV: Conversion of (A) sulphide to sulphur and (B) sulphur to sulphate

From Table 4.4 it can be observed that 10 minutes into the experiment, elemental sulphur concentration reached the highest, but afterwards sulphur was being oxidized to sulphate due to observed increase in sulphate concentration. In spite of sulphur oxidation, sulphide was oxidised. However, greater sulphur recovery was recorded (95%) at this condition compared to the previous ones (90% at pH 6.7 and Eh -130mV). Those results implied that the redox potential is the main tool in sulphur recovery thus agreeing with Xu et al finding (2012). Likewise in the previous conditions (Eh = -130 mV and pH 6.7), sulphide oxidation to elemental sulphur also fits on a polynomial curve of second degree and sulphur oxidation to sulphate has a linear trend. From Figure 4.13(A), it was observed that sulphide oxidation was unstable while Figure 4.13 (B) shows stable sulphur oxidation with a linear trend: a large amount of sulphur produced was directly converted to sulphate whereas, sulphide was oxidized but some of it was not converted to elemental sulphur.

4.4 Biological Treatment of Acid Mine Drainage in Batch Reactor

Sulphate reducing bacteria (SRB) are known to utilize carbon sources such as lactate, ethanol, acetate, lignocellulose and sawdust as electron donors during the reduction of sulphate to produce sulphide (Tang *et al.*, 2010). Studies have shown that organic matter for SRBs can be expressed in the form of a COD/sulphate ratio (Mc Cartney & Oleszkiewicz., 1993; Li *et al.*, 1996). Rinzema *et al.*, (1988) reported that a COD/sulphate ratio of 0.67 did not provide sufficient sulphate for SRBs to completely utilize organic matter. At a ratio below 0.67, the amount of organic matter was insufficient for complete sulphate reduction.

The majority of integrated sulphate removal experiments consist of two reactors: one for sulphate reducing phase and the second one for sulphide oxidizing phase. It would be an ideal to have SRB and SOB working in the same reactor. Xu *et al.*, (2012) demonstrated the technical feasibility of a single reactor (81.5% of sulphate was removed and up to 72% of sulphur was recovered. There have not been more successful studies reported regarding a single reactor. The objective of these set of experiments was to investigate the feasibility of mixed *Lysinicibacillus sp.* to recover elemental sulphur from a sulphate rich solution in a single batch reactor. Preliminary studies were first carried out to determine the suitable COD/Sulphate ratio for biogenic sulphide production.

4.4.1 Influence of COD/Sulphate Ratio on pH

pH was considered as an indicator and controller of the performance of the bacteria strains in the different batch reactors. Figure 4.14 illustrates the changes in pH values in each sample during the incubation period. It was observed that the pH uniformly increased before decreasing. The initial increase in pH can be explained by the production of alkaline species which are generated during the metabolic reaction of the bacteria (Equation 4.3). The increase in pH was thought to be beneficial for bacteria growth.

$$2CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2CH_3COO^- + 2HCO_3^- + HS^- + H^+ \quad (4.3)$$

The decrease in pH values is most likely due to the production of H_2S in the solution. At the end of the experiment, the final pH was ranging between 6.3-6.95 which was close to neutral.

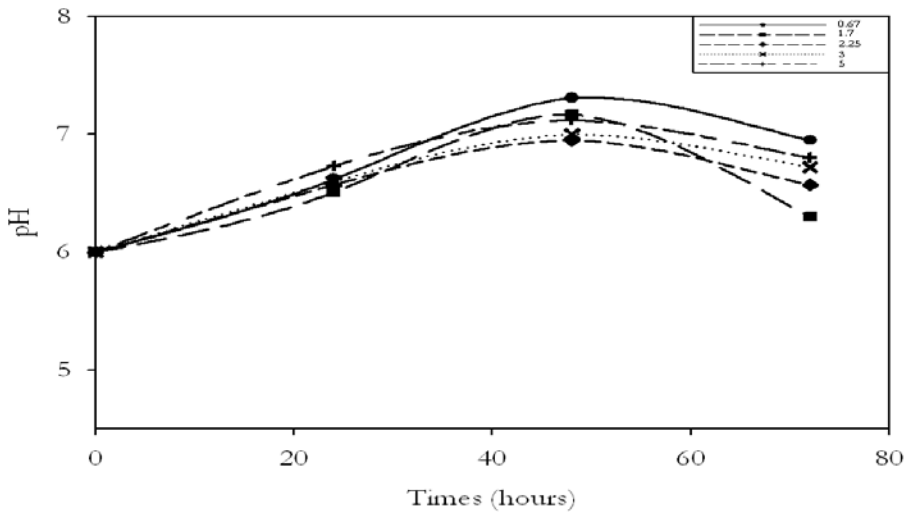

Figure 4.14: Change of pH during anaerobic culture with COD/Sulphate Ratio

4.4.2 Influence of COD/Sulphate ratio on sulphate removal rate

A high removal efficiency of sulphate indicates high bacteria activity (SRB) thus higher biogenic sulphide production, while low sulphate removal efficiency implied a decrease in SRB activity. The changes in sulphate concentration during the incubation period of the experiment are illustrated in Figure 4.15. Maximum sulphate removal rate is shown in Figure 4.16.

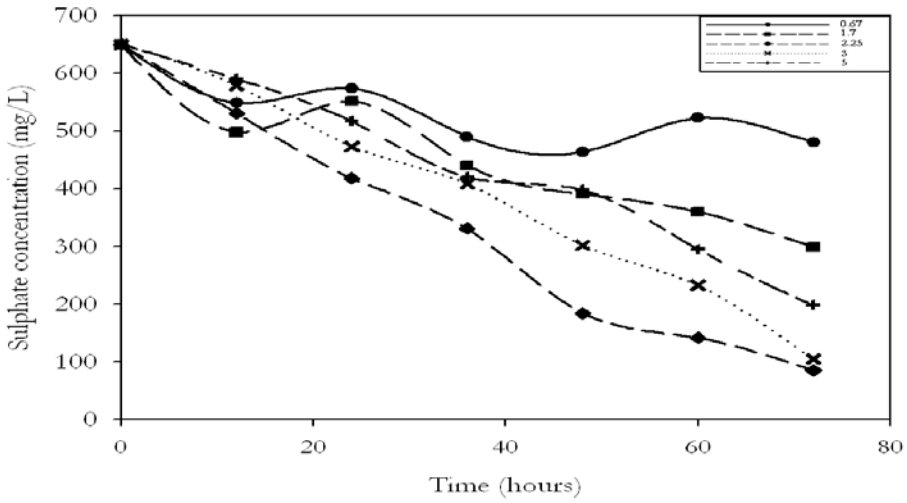

Figure 4.15: Change of Sulphate Concentrations during the batch culture with COD/Sulphate ratio

Similar to changes in pH, sulphate concentration started to decrease at 12hours and reached the lowest concentration at the end of the incubation period. At the ratio of 0.67 the concentration reached its lowest at 48 hours before attaining an increase again of 18%. Figure 4.22 shows the sulphate removal rates at the different ratios. As the ratio increased from 0.6 to 2.25, the sulphate removal also increased. It was evident that COD/Sulphate ratio of 2.25 was the optimum for the sulphate removal and growth of SRB.

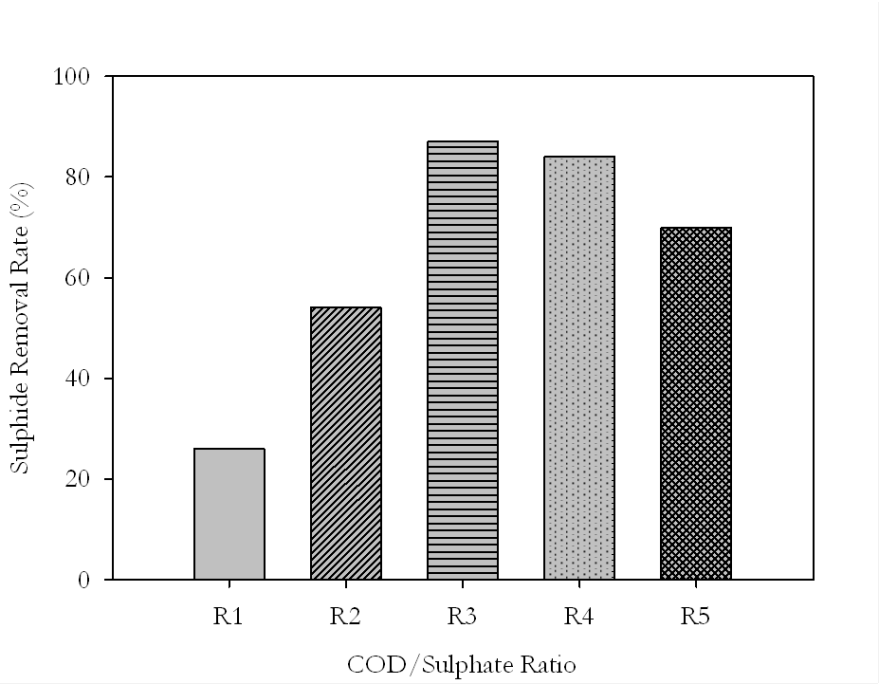

Figure 4.16: Sulphate removal rate at the different COD/Sulphate ratios

4.4.3 Influence of COD/Sulphate ratio on Biogenic Sulphide production

Figure 4.17 shows the changes in sulphide concentration throughout the incubation period with respect to COD/Sulphate ratio. Similar to the removal of sulphate, sulphide concentration increased and at the end of the experiment, sulphide concentrations were 31, 67, 119, 99 and 86 mg/L for COD/Sulphate ratio of 0.67, 1.7, 2.25, 3 and 5 respectively. According to a study by Choi and Rim (1991) COD/Sulphate ratios exceeding 2.7 could have negative effects on SRB activity. El Bahoumy *et al.*, (1999) suggested that COD/Sulphate values ranging from 1.5 to 2.5 was more than enough for sulphide production to reach maximum values when lactate is used as the carbon source. This explains the high sulphate reduction rate and sulphide production at a ratio of 2.25.

It was reported that the activity of SRB is inhibited when sulphide concentration reaches 900mg/L of total sulphide concentration of which 450mg/L are in the un-ionized form (Renze *et al.*, 1997). Therefore the sulphide produced at all samples could not have a huge effect on bacterial activity.

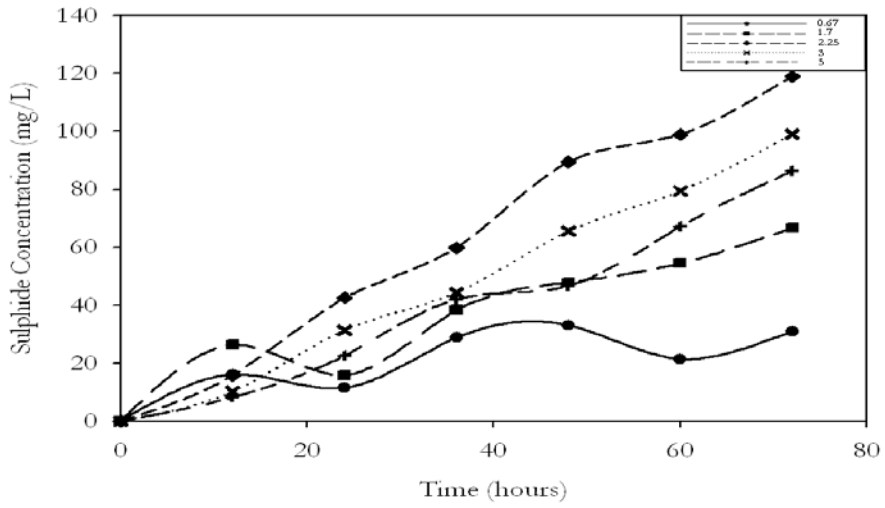

Figure 4.17: Variation of sulphide concentration over time during batch culture with COD/Sulphate ratio

4.4.4 Partial Sulphide Oxidation and Elemental Sulphur Recovery

Since the COD/Sulphate ratio of 2.25 showed the best results, the biogenic sulphide produced was used for elemental sulphur formation. At the end of the sulphate reducing phase, the mixed *Lysinicibacillus species* were inoculated into the reactor and glucose nutrient broth was also added as organic matter for the sulphide oxidizing phase to occur. Figure 4.18 shows the concentrations of sulphur species in the reactor throughout the incubation period.

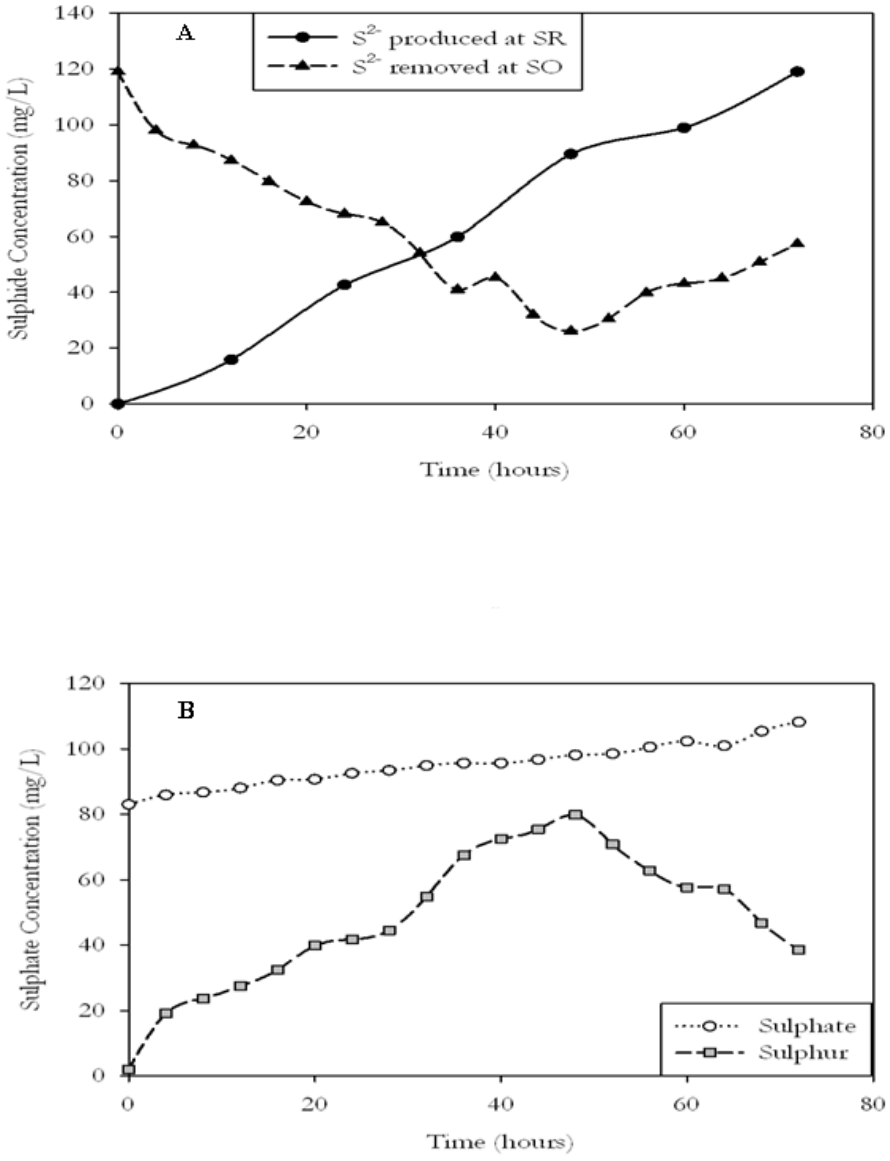

Figure 4.18: Changes of Sulphide (A), Sulphur and Sulphate (B) concentrations during the incubation period

Table 4.5: Single batch reactor performance on sulphur containing compounds

Time (minutes)		**S^{2-} (mg/L)**		**SO_4^{2-} (mg/L)**		**S^{2-} removal rate (%)**	**S^0 production (mg/L)**	**S^0 recovery (mg/L)**
initial	final	$S_{initial}^{2-}$	S_{final}^{2-}	SO_4^{2-} initial	SO_4^{2-} final			
0	4	119	98	83	85	17.6	18.1	14
4	8	98	92.7	83	85.9	5.41	2.4	↓
8	12	92.7	98.2	85.9	86.7	-	-6.3	↓
12	16	98.2	79.7	86.7	88	18.8	16.1	16
16	20	79.7	72.5	88	90.4	8.78	4.8	24
20	24	72.5	68.1	90.4	90.7	6.06	4.1	↓
24	28	68.1	65	90.7	92.5	4.55	1.3	↓
28	32	65	54	92.5	93.4	17	10.1	235
32	36	54	40.9	93.4	94.9	24.26	11.6	26
36	40	40.9	45.2	94.9	95.6	-	-5	↓
40	44	45.2	31.9	95.6	96.7	29.42	12.2	↓
44	48	31.9	26.1	96.7	98.1	18.18	4.4	↓
48	52	26.1	30.5	98.1	98.5	-	-4.8	↓
52	56	30.5	38.8	98.5	100.5	-	-10.3	148
56	60	38.8	43.1	100.5	102.3	-	-6.1	↓
60	64	43.1	45	102.3	100.9	-	-0.5	↓
64	68	45	50.8	100.9	105.4	-	-10.3	516
68	72	50.8	57.3	105.4	108.2	-	9.3	↓

Figure 4.18 (A) shows the production of sulphide and its removal over time, during the sulphate reducing (SR) phase and the sulphide oxidizing (SO) phase. Table 4.5 shows the concentration of the sulphide, sulphate and sulphur measured in the single batch reactor. It was observed that, the sulphide oxidizing phase was unsuccessful. An average of 3% sulphide removal rate with a maximum rate of 18% was recorded. It was assumed that the SOB bacterial activity was affected by the presence of SRB, as well as the low dissolved oxygen concentration (Okabe *et al.*, 2005). The presence of lactate most likely stimulated the growth of SRB (Kaksonen, 2004 and Oyekola, 2008), thereby making the SO process less efficient. This was in accord with Celis- Garcia et al (2008) findings which also demonstrated low sulphur recovery.

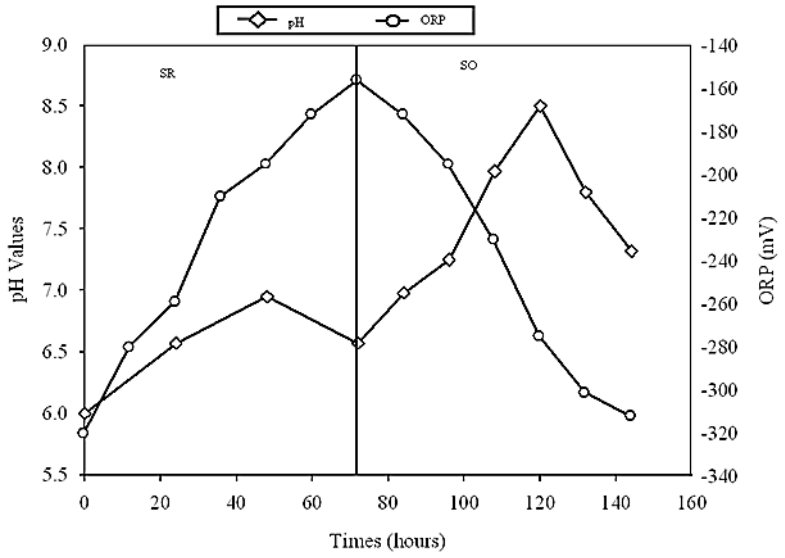

Figure 4.19: Variation of pH and Eh throughout the experiment

Figure 4.19 illustrates the changes in pH and Eh values in the integrated reactor. The highest pH reading throughout the entire experiment was 8.5. The subsequent decrease in pH can be attributed to the presence of hydrogen ions, produced from the oxidation of elemental sulphur to sulphate as followed:

$$2S^0+2OH^-+ 3O_2 \rightarrow 2SO_4^{2-} + H^+ \quad (4.4)$$

Redox potential was also affected during the production of S^0. The redox potential increased from -320mV to -156mV in the SR phase and subsequently decreased from -130mV to -312mV. This represents an inverse relationship between pH and Eh during the course of the treatment process

4.5 Chapter Summary

In the integrated reactor, 87% of sulphate was reduced to sulphide and 18% of the sulphide was measured to be left as residual but a low amount of sulphur was formed. It was concluded that the lactate present in the reactor could have stimulated the growth of SRB which affected the sulphide oxidizing reactor.

CHAPTER 5

KINETIC MODELLING

5.1 Viable Biomass of *Lysinicibacillus fusiformis* Incubated on sulphide (50-100 mg/L) under optimum conditions

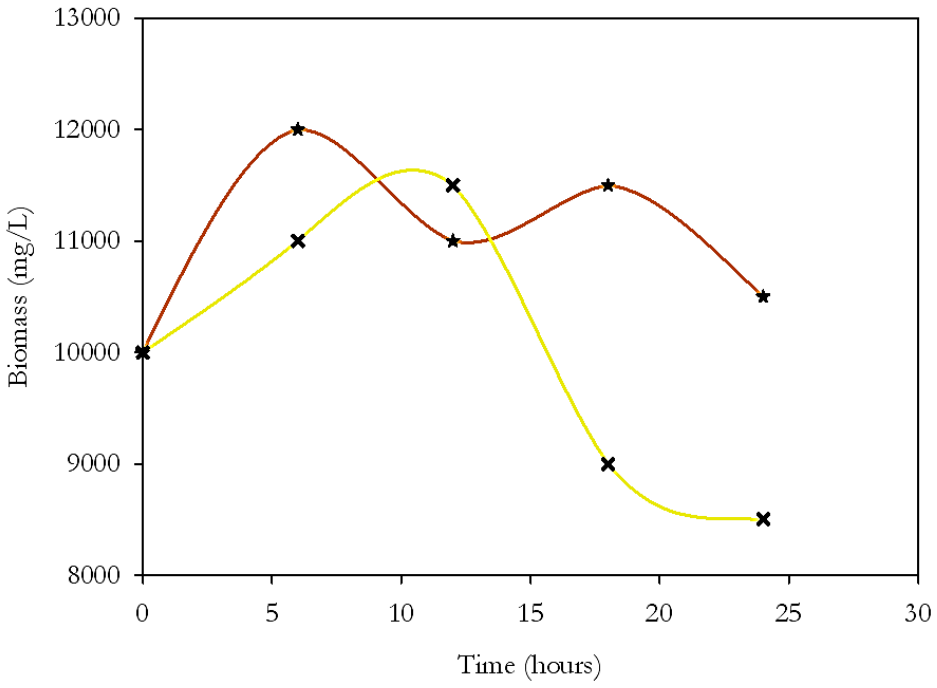

Figure 5.1: Biomass of *L.fusiformis* at sulphide concentration of: (—★—) 50 mg/L and (—✕—) 100 mg/L

At an initial concentration of 50 mg/L, the biomass reached a maximum value 12000 after 6 h of incubation, while an initial concentration of 100 mg/L showed maximal biomass of 11800 after 12 h of incubation. A decline in biomass value was observed subsequently for the separate concentrations Buisman *et al.* (1991) stated that microbial growth could not be inhibited by a sulphide concentration of 300 mg/L thus the reduction in biomass could be the result of reduction in concentration of organic matter.

5.2 Kinetic Modelling Theory

The quantitative determination of elemental sulphur formation was done by mass balance calculation (Chen *et al.*, 2009) or via sulphite method (Jiang *et al.*, 2009). Xu et al (2013) stated that kinetic models could also assist during the optimization process of elemental sulphur generation.

At low and high oxygen concentration, sulphide is oxidized to elemental sulphur (Equation 5.1) and sulphate (Equation 5.2) respectively.

$$2HS^- + O_2 \rightarrow 2S^o + 2OH^- \quad (5.1)$$

$$2HS^- + 4O_2 \rightarrow 2SO_4^{2-} + 2H^+ \quad (5.2)$$

According to Eq. (5.1), sulphur production depends on sulphide ion (HS^-) and dissolved oxygen. Dutta (2008) stated that when two substances limit the biological reaction rate, the rate of the product adopted is the following:

$$r = \frac{C_e \times k_1 \times [HS^-]}{k_2 + [HS^-]} \times \frac{O_2}{k_3 + O_2} \quad (5.3)$$

where: C_e = Optical density of the bacteria, k_1= reaction rate constant (mg L^{-1} h^{-1}), k_2= reaction rate constant (mg L^{-1}), k_3= reaction rate constant (mg L^{-1}), $[HS^-]$= concentration of sulphide (mg/L) and O_2= concentration of the dissolved oxygen (mg/L).

However, redox potential was used as the oxygen control, therefore the dissolved oxygen concentration was ignored (see Eq. 5.4).

$$r = \frac{X \times k_1 \times [HS^-]}{k_2 + [HS^-]} \quad (5.4)$$

Analogous to the Monod kinetics, k_1 is analogous to the maximum conversion rate (k_m) divided by the yield (Y), C_e is analogous to the biomass concentration (X) and k_2 is analogous to K half saturation constant.

$$r = \frac{km}{Y} \times \frac{[HS^-]}{K + [HS^-]} X \quad (5.5)$$

Monod kinetics has been used by several researchers (Alcantara *et al.*, 2004; Gadekar *et al.*, 2006; Ni *et al.*, 2012 and Xu *et al.*, 2013). It was proposed as an appropriate expression to highlight substrate utilisation during elemental sulphur formation. The model also highlights that the rate and the extent to which sulphide oxidation in a bacterial system is affected by the biomass concentration and endogenous decay rate represented by the term k_d. This indicates that the substrate consumed by the bacteria provides the energy required for growth, reproduction and metabolic function. Therefore, the active biomass concentration is assumed to increase with the decreasing concentration of substrate. Therefore at t=0, the substrate concentration is high and the biomass concentration is low, the rate of decay of the bacteria is then written as Equation 5.6 and when the substrate is exhausted, the rate of decay is expressed as Equation (5.7).

$$X = X_0 e^{(km - kd)t} \quad (5.6)$$

$$X = X_0{}^{e^{-kdt}} \quad (5.7)$$

Where: k_d= endogenous decay rate (time^{-1}), X_o = initial biomass concentration (mg/L), k_m = maximum specific sulphide oxidation rate (time^{-1}).

However during the optimal condition investigations, the bacterial cells were harvested thus their concentration was kept constant throughout the experimental phase. So Equation 5.5 was written as:

$$r = \frac{km}{Y} \times \frac{[HS^-]}{K + [HS^-]} \quad (5.8)$$

Sulphide Oxidation involves two main processes which were illustrated in Equations 5.1 and 5.2. Both processes were incorporated into the above model (Equation 5.8).

Process 1: Sulphide conversion to elemental sulphur

$$r = \frac{km^{a}}{Y^{a}} \times \frac{[HS^{-}]}{K^{a} + [HS^{-}]} \tag{5.9}$$

Process 2: Sulphur conversion to sulphate

$$r = \frac{km^{b}}{Y^{b}} \times \frac{[S^{0}]}{K^{b} + [S^{0}]} \tag{5.10}$$

5.3 Parameter Evaluation

The unknown parameters defined based on the equations, and the type of systems used to produce elemental sulphur, were k_m, K_c and Y in each process. These paramters were determined by performing a nonlinear regression analysis using Aquasim (Riechert, 1998). For each parameter, a search was performed through a range of values. Trial values of the unknown parameters were initially investigated. It was also required to impose some constraints, in order to set upper and lower limits for each parameter so that invalid values were not omitted. Whenever optimization converged at/or very close to a constraint, the latter was relaxed until the constraint no longer forced the model. The processes were repeated till unique values for each parameter, lying away from the constraints but between the set of limits were determined. The best fit values were found through repetition of parameter estimation of the unknown. The objective function for parameter optimization was defined as the least sum of the squares between the observed and the modelled concentrations as follows:

$$\sigma^{2} = \frac{1}{n-q}\sum_{i=1}^{i=n}(y_{i} - y)^{2} \tag{5.11}$$

Where: σ= average deviation of model from the measured values; y_i = observed variables; y= simulated variables; n= number of observations and q= degrees of freedom representing the number of parameters being evaluated.

The batch reactor used was a volumetric flask of 250mL and a working volume of 100mL. The initial concentration of sulphide ions (S^{2-}) was of 50 or 100mg/L. Oxygen was purged into the reactor to give an initial ratio of 2:1 (sulphide:oxygen). The temperature was kept constant throughout the test at 30±1°C and the initial pH was 8.

5.3.1 Kinetic Parameter Estimation

Experimental data with sulphide concentration of 50 mg/L at pH 6.7 and 8 and Eh = -130 and -80 mV respectively were used to estimate the kinetic parameters k_m, K_c and Y in each process. The validation of both models was performed and Figure 5.2 confirms that the kinetic parameters values were obtained under the different conditions (See Table 5.1).

Table 5.1: Optimum Kinetic Parameter in Biological Sulphide Oxidation

	Sulphide Oxidation				**Sulphur Oxidation**			
	$k_m{}^a$	K^a	Y^a	χ^2	$k_m{}^b$	K^b	Y^b	χ^2
pH 6.7 and Eh= -130mV	10	0.010	3.00	14.74	746	8401	6.85	13.04
pH 8 and Eh= -80mV	126	564.73	0.377	37.72	682	9598	10.3	21.63

As the Oxidation reduction potential increased, the half saturation (*K)* constants were observed to increase whereas the yield coefficients (*Y*) were decreasing but the maximum conversion rate (k_m) seemed to decrease during the sulphur oxidation stage. χ^2 also increased as Eh increased but the parameters were more accurate during the sulphur oxidation stage since χ^2 were smaller. Those results implied that ORP was the main parameter affecting sulphur formation process thus confirming earlier findings.

During the sulphide oxidation stage, at pH 6.7 and Eh= -130 mV, k_m was observed to be similar and higher to those reported in Gadekar *et al.*, (2006) and Xu *et al.* (2013) respectively. The half saturation constant was lower to values reported by Alcantara *et al.*, (2004); Gadekar

et al.,(2006) and Xu *et al.*, (2013) but higher under optimal conditions and the Yield coefficient was higher than Xu et al., (2013).

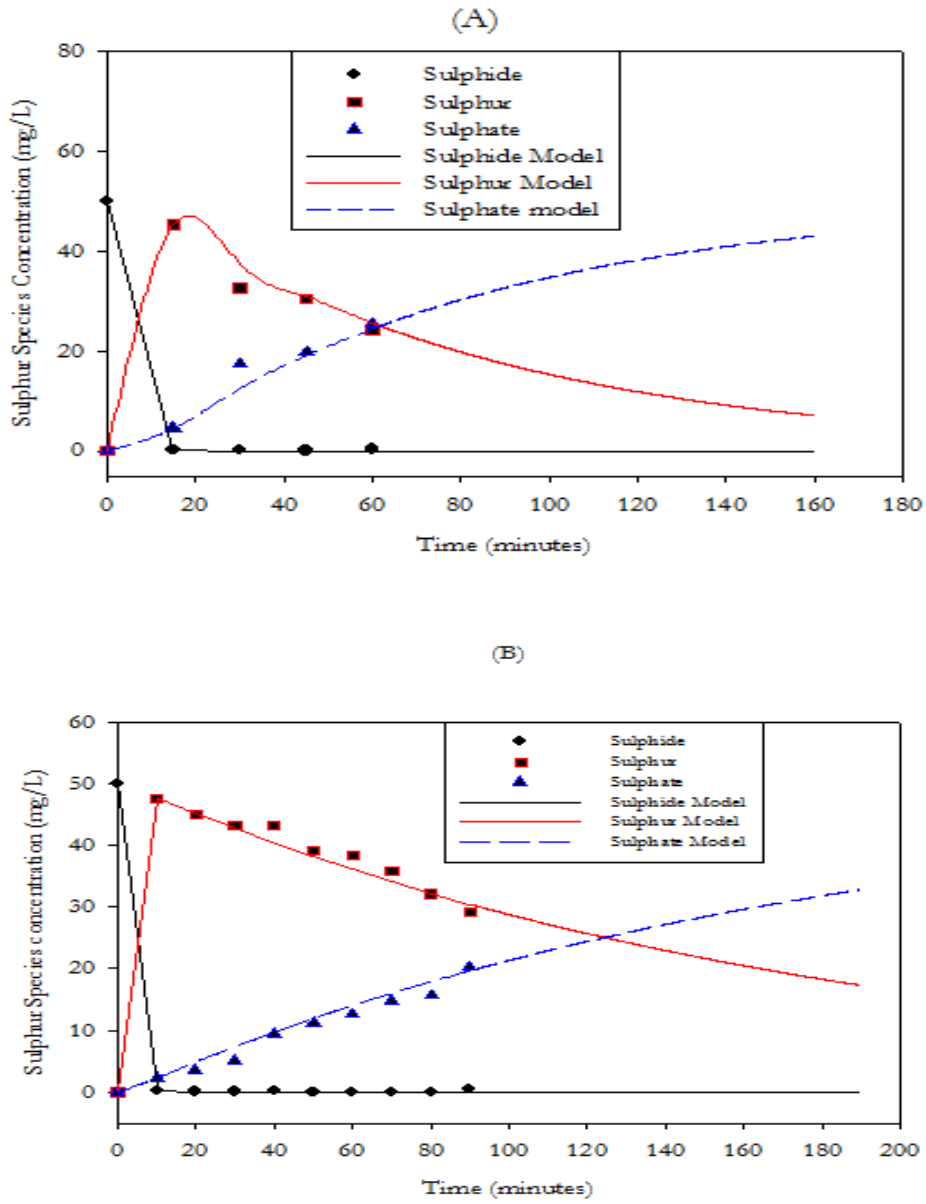

Figure 5.2: Sulphur species oxidation in batch cultures at (A) pH 6.7 and Eh -130mV and (B) pH 8 and Eh -80mV.

Strigul et al., (2009) indicated that in a Monod model, a stationary phase is produced and it is infinite, this was observed in Figure 5.2, as the sulphur and sulphate concentrations reached a stationary phase. Strigul et al (2009) also revealed that the microbial concentration in the stationary phase determines the yield coefficient, since the microbial concentration was

unknown but the yield was estimated at each process it was deduced that microbial concentration was higher in the sulphur oxidation but it was at its lowest at the sulphide oxidation stage under optimal conditions meaning the biomass concentration was increasing.

From Figure 5.2, It was noted that sulphide will be eliminated from the system but in Figure 5.2 (A) only a few amount (around 5 mg/L) of elemental sulphur will be left in the flask whereas the entire sulphur species will be in the form of sulphate and in Figure 5.2 (B) around 20mg/L of elemental sulphur will be left as a residual while sulphate will be the main sulphur species formed.

5.3.2 Sensitivity Analysis

The sensitivity analysis combines the tasks of identifiability analysis and uncertainty analysis. The latter will not be discussed in this study. Identifiability analysis aims on verifying if the parameters can be determined using the measured data during the experimental phase and it also focuses on estimating the uncertainty of the parameters estimates. Additional information of the identifiability analysis is the sensitivity functions which are written below:

$$\delta_{y,p}^{a,a} = \frac{\partial y}{\partial p} \tag{5.12}$$

$$\delta_{y,p}^{r,a} = \frac{1}{y}\frac{\partial y}{\partial p} \tag{5.13}$$

$$\delta_{y,p}^{a,r} = p\frac{\partial y}{\partial p} \tag{5.14}$$

$$\delta_{y,p}^{r,r} = \frac{p}{y}\frac{\partial y}{\partial p} \tag{5.15}$$

With y is an arbitrary variable calculated by Aquasim (2.0) and p is the model parameter represented by a constant variable or by a real list variable. The absolute-absolute sensitivity function (Equation 5.9) measures the absolute change in y per unit of change in p. The relative-absolute sensitivity function (Equation 5.10) measures the relative changes in y per unit of change in p. the Absolute-relative sensitivity function (Equation 5.11) measures the

absolute change in y for 100% change in p and the relative-relative sensitivity function (Equation 5.12) measures the change in y for a 100% change in p. all the changes are calculated in linear approximation only. However, the most useful sensitivity functions are the absolute-relative and relative-relative sensitivity functions because their unit do not depend on the unit of parameter but the absolute-relative sensitivity function is preferred over the relative-relative one because this latter does not give useful results when the value of y becomes small during simulation. For this reason the absolute-relative sensitivity function was used for simulation.

Figure 5.3 illustrates the sensitivity function of sulphur species concentration under the optimized condition with respect to k_m, K and Y at each process was analysed to compare the effect of each parameter on the oxidation process. It was observed that k_m, Y and K of the sulphur oxidation step do not affect the sulphide oxidation stage process, it was not shown in the Figure 5.3 (A) but for the first 15 min the model was highly sensitive to k_m^a, Y^a and K^a before no more sensitivity was recorded. In Figure 5.3 (B) each parameters, no matter the stage, affects the model: for the first 15 min, Y^a, k_m^a and K^a affect the model whereas the sulphur concentration was highly sensitive to k_m^b, Y^b and K^b the entire process. In Figure 5.3 (C) sulphate concentration was highly sensitive to k_m^b, Y^b and K^b but was not sensitive to k_m^a, Y^a and K^a. This confirmed the above findings that sulphate is directly converted from sulphur. The above results were on agreement with the one reported by Xu et al., (2013)

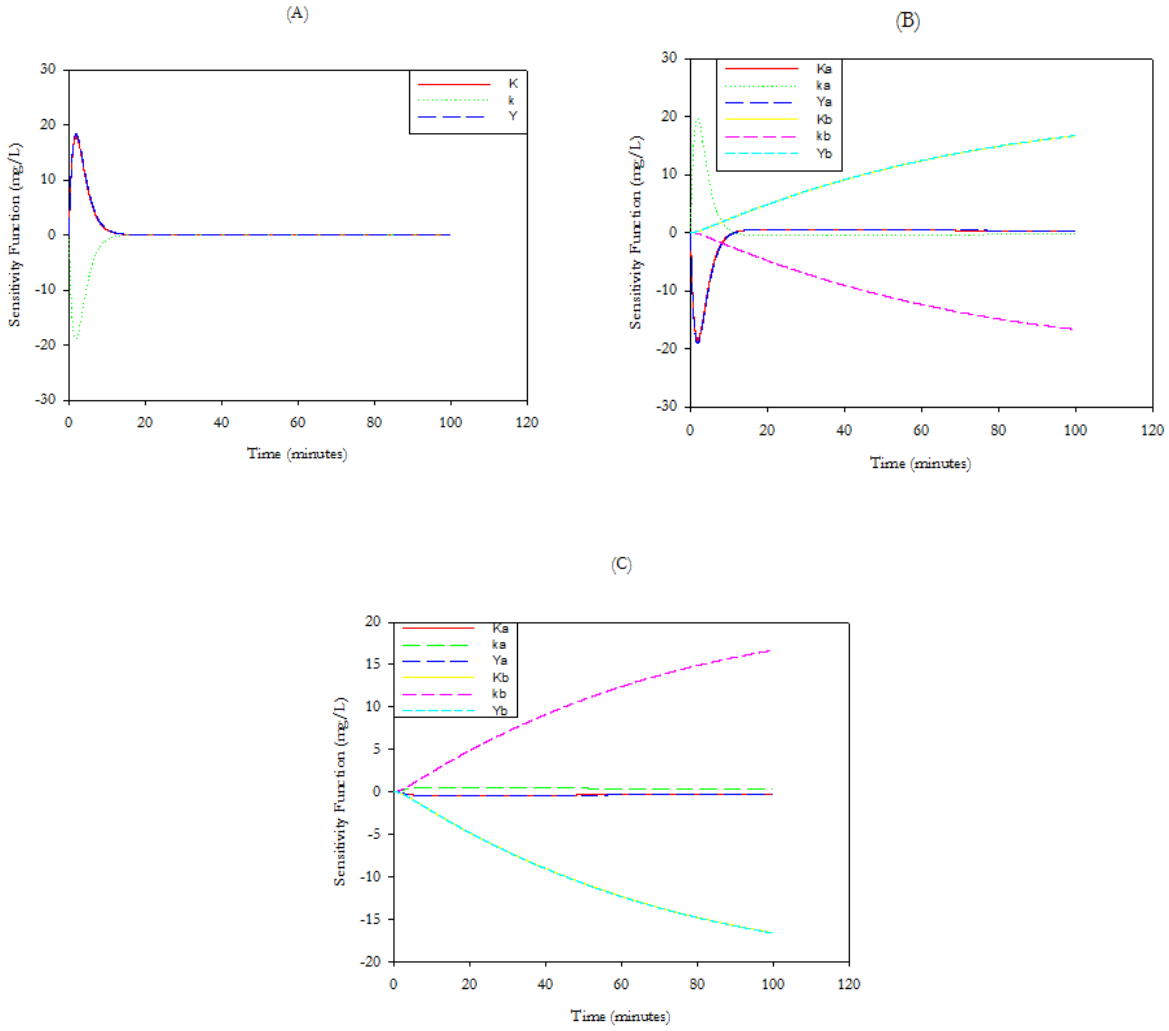

Figure 5.3 Sensitivity test for the sulphide oxidation and sulphur oxidation processes under optimal conditions (A) sulphide concentration, (B) sulphur concentration and (C) sulphate concentration.

5.4 Chapter Summary

The kinetics of sulphide oxidation in batch culture by *L. fusiformis* species can be described using the Monod model. The model indicates that the sulphur oxidation stage it is a stable process and yield is produced at a higher concentration at this stage compared to the sulphide oxidation to sulphur phase. Aquasim 2.0 was able to simulate the removal of

sulphide under optimal conditions in a batch reactor and it was adequate in modelling the system. These results would allow for the development of a more predictive model and allow for accurate prediction of the overall performance of the reactor.

The Monod model was observed to be an appropriate fit for the data obtained. The obtained parameters were comparable to parameters found in earlier literature. They can be incorporated into reactive transport models used for the design and operation of sulphide remediation systems (as well as sulphide recovery process).

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Six microorganisms, *Micrococcus luteus, Lysinicibacillus fusiformis, Lysinicibacillus sphaericus, Pseudomonas putida, Fusarium oxysporum and Penicillium simplicissimum* were isolated a mining environment to evaluate their effectiveness in removing sulphide and to select the most efficient cultures. The criterion for selection was based on: the culture with the highest sulphide removal rate was selected as the most suitable strain for the rest of the experiment. *Lysinicibacillus fusiformis* was regarded as the most efficient not only due to its high rate but the bacterium was observed to be able to remove sulphide in the presence and the absence of carbon source.

Partial oxidation was reported to occur within strict conditions. The effects of pH, redox potential and presence of a carbon source on the oxidation of sulphide to produce elemental sulphur was investigated. It was observed that the pH of the solution did not have an effect on the bacterial ability to remove sulphide. At a pH range of 6-8; the same sulphide removal rate was observed. The redox potential was considered as the main key parameter in this study. It was observed at Eh of around -80mV partial sulphide oxidation does occur and sulphur is produced.

The results obtained from this study highlight the feasibility of elemental sulphur production through partial sulphide oxidation and serve as a foundation for further studies towards the development of cost-effective sulphur recovery procedure from AMD. Batch modelling results showed that partial sulphide oxidation fitted well the Monod kinetic model rate for biological process. The kinetics parameters developed could be used to design a bioreactor for sulphide removal system.

6.2 Recommendations

In order to achieve optimum application for this particular technology it will be needed to find a cheaper and efficient organic matter or evaluate the feasibility and efficiency of the organic matter left during biological sulphate reducing process in order to reduce the cost.

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APPENDIXES

Appendix A

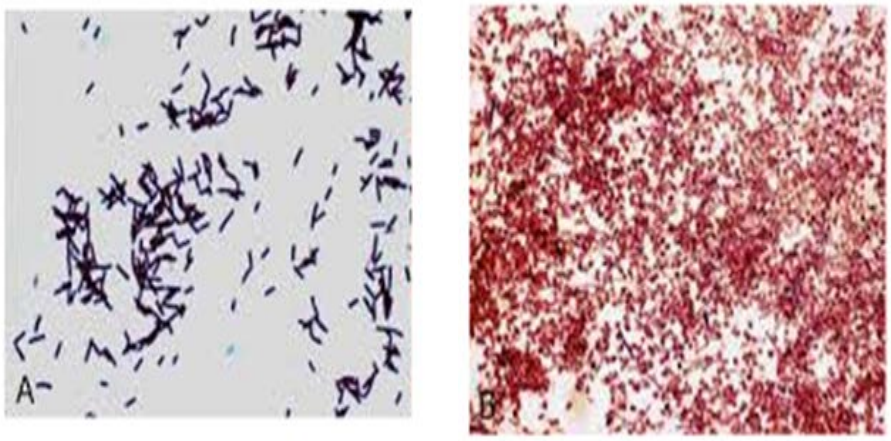
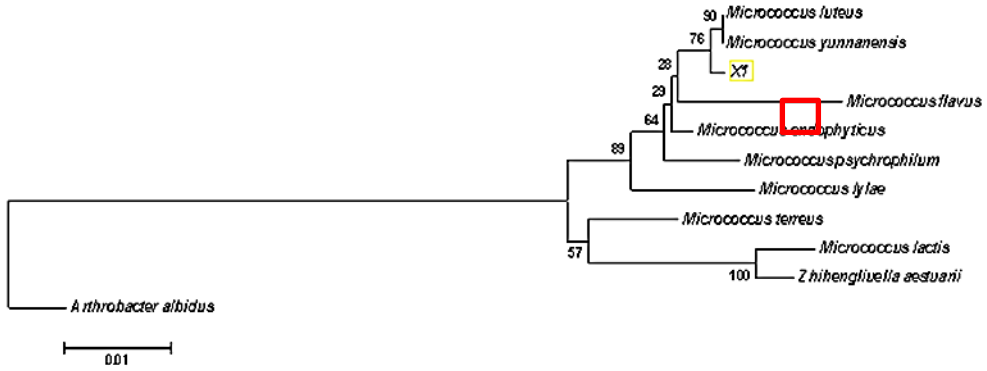
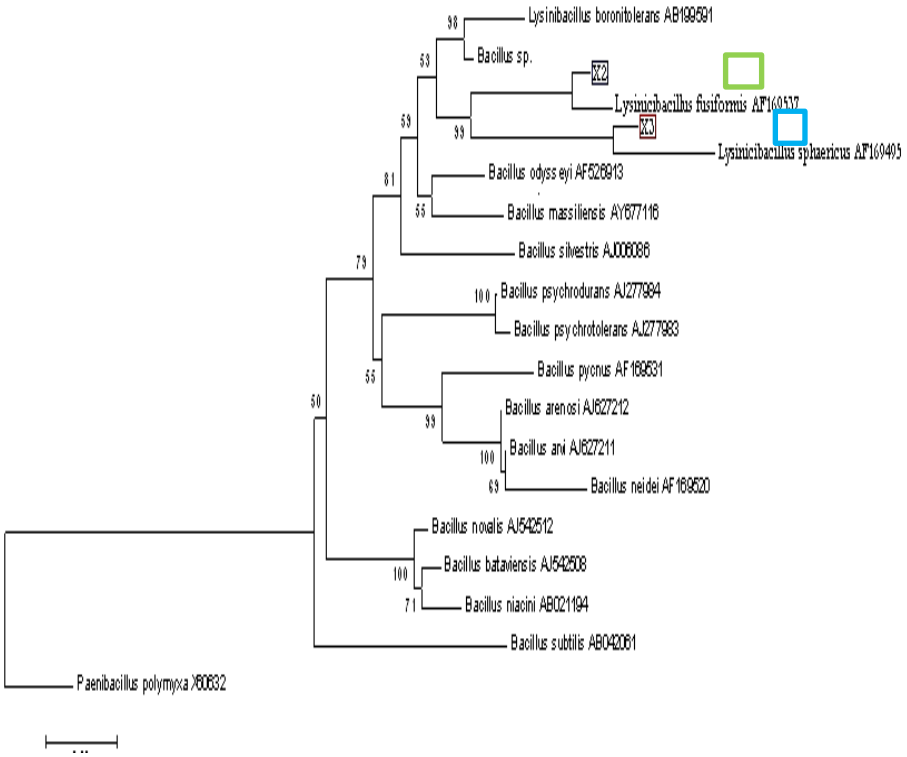


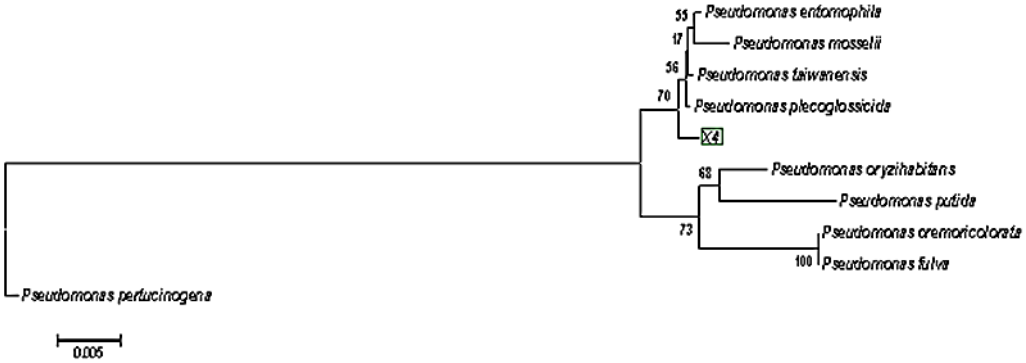
Figure 1: (A) Gram positive bacterial species and (B) Gram negative bacterial species.



(A):



(B):



(C):

Figure 2: Phylogenetic tree diagram showing: (A)sample X1 identified as a homology of *Micrococcus yunnanensis* and *Micrococcus luteus*, (B) sample X2 and X3 identified as a homologs of *Lysinicibacillus fusiformis* AF169537 and *Lysinicibacillus sphaericus* AF169465 respectively and (C) sample X4 identified as a homology of *Pseudomonas plecoglossicida.*

APPENDIX B

****

AQUASIM Version 2.0 (win/mfc) - Parameter Estimation File

****

Number of parameters = 4
Number of data points = 5
Estimation method = secant

Parameters:

Name	Unit	Start	Minimum	Maximum
C_Aini1	mg/L	50	0	1000
K_A	mg/L	0.0101684	0.01	1000
u_A	1/minutes	10	0.01	1000
Y_A		3.00279	0.01	10

Calculations:

C_Aini1 [mg/L]	K_A [mg/L]	u_A [1/minutes]	Y_A []	Chi^2
50	0.0101684	10	3.00279	14.7403
60	0.0101684	10	3.00279	9957.72
50	10.0101	10	3.00279	17400.7
50	0.0101684	19.9999	3.00279	15.9488
50	0.0101684	10	3.10269	263.915
50	2.34889	2.98189	2.80299	165819
50	0.170698	29.9998	3.00275	15.9488
55	0.0904333	19.9999	3.00277	27.0108
50	0.01	25.48	3.00177	15.9488
52.5	0.0502166	22.7399	3.00227	18.7143
50	0.01	34.8212	3.00003	15.9488
51.25	0.0301083	28.7805	3.00115	16.6402
50	0.0101614	39.6584	2.99727	15.9488
50.625	0.0201349	34.2195	2.99921	16.1217
50	0.0111273	9.96316	2.99175	14.7423
50.3125	0.0156311	22.0913	2.99548	15.9921
50	0.0120854	9.92632	2.98072	14.7508
50.1563	0.0138582	16.0088	2.9881	15.9596
50	0.0137385	9.85263	2.95864	14.7804
50.0781	0.0137984	12.9307	2.97337	15.9515
50	0.0101684	10	3.00279	14.7403

Parameter estimation successfully finished (convergence criterion met)

C_Aini1	K_A	u_A	Y_A
[mg/L]	[mg/L]	[1/minutes]	[]

Estimated values of the parameters:

50	0.0101684	10	3.00279

Standard errors could not be estimated

Contribution of data series to Chi^2:

Calculation:	Data Series:	Chi^2 ini:	Chi^2 end:
fit1	Cmeas_A1	14.7403	14.7403
		---	---
		14.7403	14.7403

Number of steps performed = 8
Number of simulations performed = 21

****

AQUASIM Version 2.0 (win/mfc) - Sensitivity Analysis File

****

Ranking of mean absolute sensitivities and error contributions:

Calculation Number: 1
Compartment: Reactor
Zone: Bulk Volume

Variable: C_A

	Parameter:	Sens AR: [mg/L]	Parameter:	Error Contr.: [mg/L]
1	C_Aini1	7.562	C_Aini1	0.7562
2	u_A	3.753	Y_A	0.2499
3	Y_A	3.751	u_A	0.07507
4	K_A	0.001521	K_A	0.02992
5	K_B	1.825e-006	Y_B	4.646e-008
6	Y_B	1.705e-006	u_B	4.849e-010
7	u_B	1.701e-006	K_B	4.913e-011
8	C_Aini2	0	C_Aini2	0
9	Cmeas_A2	0	Cmeas_A2	0
10	Cmeas_B1	0	Cmeas_B1	0
11	Cmeas_B2	0	Cmeas_B2	0
12	Cmeas_C1	0	Cmeas_C1	0
13	Cmeas_C2	0	Cmeas_C2	0
14	Cmeas_A1	0	Cmeas_A1	0

Variable: C_B

	Parameter:	Sens AR: [mg/L]	Parameter:	Error Contr.: [mg/L]
1	C_Aini1	25.91	C_Aini1	2.591
2	u_B	12.96	Y_A	0.3895
3	Y_B	12.94	Y_B	0.3526
4	K_B	12.91	u_A	0.117
5	u_A	5.85	K_A	0.04748
6	Y_A	5.848	u_B	0.003695
7	K_A	0.002414	K_B	0.0003475
8	C_Aini2	0	C_Aini2	0
9	Cmeas_A2	0	Cmeas_A2	0
10	Cmeas_B1	0	Cmeas_B1	0
11	Cmeas_B2	0	Cmeas_B2	0
12	Cmeas_C1	0	Cmeas_C1	0
13	Cmeas_C2	0	Cmeas_C2	0
14	Cmeas_A1	0	Cmeas_A1	0

Variable: C_C

	Parameter:	Sens AR:	Parameter:	Error Contr.:

		[mg/L]		[mg/L]
1	C_Aini1	16.53	C_Aini1	1.653
2	u_B	12.96	Y_B	0.3526
3	Y_B	12.94	Y_A	0.1707
4	K_B	12.91	u_A	0.05114
5	Y_A	2.563	K_A	0.0204
6	u_A	2.557	u_B	0.003695
7	K_A	0.001037	K_B	0.0003475
8	C_Aini2	0	C_Aini2	0
9	Cmeas_A2	0	Cmeas_A2	0
10	Cmeas_B1	0	Cmeas_B1	0
11	Cmeas_B2	0	Cmeas_B2	0
12	Cmeas_C1	0	Cmeas_C1	0
13	Cmeas_C2	0	Cmeas_C2	0
14	Cmeas_A1	0	Cmeas_A1	0

APPENDIX C

****

AQUASIM Version 2.0 (win/mfc) - Parameter Estimation File

****

Number of parameters = 4
Number of data points = 5
Estimation method = secant

Parameters:

Name	Unit	Start	Minimum	Maximum
C_Aini1	mg/L	47.429	0	1000
K_B	mg/L	8401.28	0.01	100000
u_B	1/minutes	746.107	0.01	10000
Y_B		6.85016	0.01	10

Calculations:

C_Aini1 [mg/L]	K_B [mg/L]	u_B [1/minutes]	Y_B []	Chi^2
47.429	8401.28	746.107	6.85016	13.0447
57.429	8401.28	746.107	6.85016	221.719
47.429	9401.28	746.107	6.85016	22.6472
47.429	8401.28	846.107	6.85016	27.9003
47.429	8401.28	746.107	6.95006	13.1467
47.0539	7868.37	710.173	7.04996	13.1431
45.9859	7521.02	583.533	6.45056	14.0653
47.5337	10401.3	900.187	6.64306	13.0567
47.4294	8220.21	658.934	6.05096	13.5554
47.4275	12401.3	1058.44	6.55146	13.1037
47.4262	16401.3	1369.03	6.25275	14.046
47.4247	24326.6	1991.95	5.66093	24.8564
47.4792	17363.9	1446.07	6.15199	14.8697
47.5294	10344.5	951.222	6.65248	16.5604
47.6298	12459.6	1271.13	6.44545	48.3005
47.8305	8208.94	1911.13	6.67785	1023.32
48.2319	15455.6	1072.62	5.87746	26.7188
48.3751	22509.9	1470.2	5.2667	19.3807
49.3211	33585.8	2030.01	3.90612	24.9269
50.1235	43523.3	3313.91	2.75422	509.266
51.7706	68826.3	5409.65	0.01	4372.51
51.1899	73106	5729.1	0.01	4372.48
38.746	52358.6	4421.22	8.36174	85.4183

30.0629	96122.7	7874.8	9.86667	383.731
29.2217	100000	7652.15	10	399.99
36.2479	44585.2	0.01	10	369.14
46.8464	0.01	0.01	7.75457	1481.15
47.3481	0.01	0.01	7.51117	1559.44
47.2004	100000	0.01	0.988563	1546.5
47.1516	0.01	791.352	0.01	4393.01
46.2638	15880	726.23	7.10733	239.908
49.7596	67991.5	138.157	1.27652	1446.72
48.5538	33995.7	69.0835	4.39385	1613.2
51.5147	0.01	0.01	4.38086	2283.95
45.9371	100000	740.451	1.91173	520.042
46.5444	50000	765.902	0.960866	70.4856
46.6408	100000	628.321	0.01	4258.14
50.572	78252.6	0.01	0.01	2123.94
51.3442	70270.9	0.01	0.01	2267.46
46.6288	19620.5	0.01	6.36888	1457.58
42.2983	100000	563.27	4.31954	628.536
44.4696	100000	595.795	2.16477	575.144
37.1675	6285.83	866.95	2.81525	2316.37
51.1362	0.01	494.633	1.68786	4393
34.3258	100000	1091.97	3.86878	126.807
26.8665	78942.5	1437.83	5.01335	343.561
8.29616	78281.8	2129.56	8.02877	2607.52
49.3242	100000	269.323	10	1806.58
50.2302	50000	381.978	5.84393	1605.15
47.2631	100000	644.563	10	1359.76
47.251	0.01	418.382	10	4393.01
43.5436	0.01	0.01	5.94103	1019.2
55.1999	51255	166.829	9.29573	2893.35
37.8436	100000	1280.01	8.49514	295.334
28.2582	74665.6	1702.89	5.20603	297.812
42.4204	76266	1011.27	10	635.717
43.8978	100000	1215.83	10	800.122
47.1477	100000	0.01	7.23539	1538.19
47.6589	54517.3	58.9623	0.55047	1062
47.7319	2052.22	0.01	10	1631.75
47.9459	100000	371.145	10	1545.92
47.1373	0.01	686.323	10	4393.01
47.4576	0.01	806.166	10	4393.01
46.3953	3858.16	342.644	6.86932	15.1691
47.0636	2955.19	171.327	8.43466	245.743
46.9355	25203.8	609.246	7.14761	687.073
46.9996	14079.5	390.286	7.79114	662.604
49.4965	14962.1	1503	7.10779	15.3756
47.7198	0.01	401.958	10	4393.01
47.5887	0.01	604.062	10	4393.01
51.5639	20944.2	584.85	7.53095	1113.64
39.1594	14989.3	441.364	5.73854	97.2955

40.3707	26402.3	2259.9	5.37186	262.404
30.8898	8314.6	386.387	3.55227	622.866
37.2399	0.01	449.613	10	4393.01
50.9048	43800.5	3773.68	8.06744	123.423
56.6228	0.01	390.058	10	4392.96
48.4968	13201.2	1324.98	7.68593	14.8691
27.0508	4853.32	425.541	6.38706	966.12
88.1855	6214.63	551.916	7.65105	4260.18
66.2777	52224.9	4669.66	10	1559.16
64.3869	34625.6	3249.54	10	1228.06
43.4079	5675.52	968.904	10	196.925
35.2294	5264.42	697.223	8.19353	539.104
42.2558	10445.6	58.9623	0.55047	27.929
43.1274	96048.5	6965.95	0.305076	4011.92
77.8698	97936.4	8361.67	10	3572.26
71.1283	66281	5805.6	10	2294.73
50.1143	0.01	482.06	10	4392.97
38.9059	10504.3	0.01	0.01	538.132
38.9933	5089.45	0.01	3.2027	559.865
43.3445	10667.6	0.01	0.01	983.223
60.6071	54302	4180.84	5.005	188.744
45.8616	10766.3	0.01	0.01	1316.34
47.2184	15024.9	760.655	7.07793	222.631
48.5554	0.01	200.245	0.01	4393.01
39.26	5630.08	500.003	4.84861	446.285
31.091	13182.3	503.483	5.93865	238.661
38.4763	11974.3	251.747	2.97433	55.1304
14.753	16106.1	617.951	9.02023	1607.31
0	20212.9	528.214	10	4393.01
50.3509	663.83	259.453	0.55047	4175.32
71.4816	0.01	397.229	0.01	4393.01
49.9468	0.01	489.241	2.87745	4393
10.9115	0.01	827.555	10	4393.01
0	8700.33	651.519	10	4393.01
34.626	25203.8	544.705	7.70043	128.853
0	32658.6	406.873	10	4393.01
4.29916	41180.2	67.6393	6.86091	3289.64
47.3393	19481.4	58.9623	0.55047	400.476
49.7077	73959.1	281.041	9.10452	1820.25
49.2701	100000	90.1246	10	1861.07
47.5866	100000	89.0652	10	1579.33
25.9429	70590.1	78.3522	8.43045	277.821
47.6036	0.01	0.01	4.44365	1593.01
47.6326	0.01	0.01	4.48339	1597.84
47.2225	0.01	729.958	10	4393.01
47.8422	6962.25	750.482	7.38969	22.5093
47.7144	53481.1	419.773	8.69484	1350.42
48.2553	6172.74	651.886	7.68569	13.8999
47.9849	29826.9	535.83	8.19027	1047.13

47.429 8401.28 746.107 6.85016 13.0447

100 steps performed (not yet converged)

Contribution of data series to Chi^2:

Calculation:	Data Series:	Chi^2 ini:	Chi^2 end:
fit2	Cmeas_B1	13.0447	13.0447
		13.0447	13.0447

Number of steps performed = 100
Number of simulations performed = 122

****

AQUASIM Version 2.0 (win/mfc) - Sensitivity Analysis File

****

Ranking of mean absolute sensitivities and error contributions:

Calculation Number: 1
Compartment: Reactor
Zone: Bulk Volume

Variable: C_A

	Parameter:	Sens AR: [mg/L]	Parameter:	Error Contr.: [mg/L]
1	C_Aini1	6.807	C_Aini1	0.7176
2	Y_A	3.376	Y_A	0.2248
3	u_A	3.369	u_A	0.06738
4	K_A	0.001441	K_A	0.02834
5	K_B	8.003e-007	Y_B	2.255e-008
6	Y_B	7.724e-007	u_B	2.016e-010
7	u_B	7.52e-007	K_B	1.905e-011
8	C_Aini2	0	C_Aini2	0
9	Cmeas_A2	0	Cmeas_A2	0
10	Cmeas_B1	0	Cmeas_B1	0
11	Cmeas_B2	0	Cmeas_B2	0
12	Cmeas_C1	0	Cmeas_C1	0
13	Cmeas_C2	0	Cmeas_C2	0
14	Cmeas_A1	0	Cmeas_A1	0

Variable: C_B

	Parameter:	Sens AR: [mg/L]	Parameter:	Error Contr.: [mg/L]
1	C_Aini1	24.61	C_Aini1	2.594
2	u_B	12.39	Y_B	0.3611
3	Y_B	12.37	Y_A	0.3528
4	K_B	12.34	u_A	0.1059
5	Y_A	5.298	K_A	0.04519
6	u_A	5.296	u_B	0.003321
7	K_A	0.002297	K_B	0.0002938
8	C_Aini2	0	C_Aini2	0
9	Cmeas_A2	0	Cmeas_A2	0
10	Cmeas_B1	0	Cmeas_B1	0
11	Cmeas_B2	0	Cmeas_B2	0
12	Cmeas_C1	0	Cmeas_C1	0
13	Cmeas_C2	0	Cmeas_C2	0
14	Cmeas_A1	0	Cmeas_A1	0

Variable: C_C

	Parameter:	Sens AR: [mg/L]	Parameter:	Error Contr.: [mg/L]

1	C_Aini1	16.02	C_Aini1	1.688
2	u_B	12.39	Y_B	0.3611
3	Y_B	12.37	Y_A	0.1547
4	K_B	12.34	u_A	0.04645
5	Y_A	2.323	K_A	0.01942
6	u_A	2.322	u_B	0.003321
7	K_A	0.0009875	K_B	0.0002938
8	C_Aini2	0	C_Aini2	0
9	Cmeas_A2	0	Cmeas_A2	0
10	Cmeas_B1	0	Cmeas_B1	0
11	Cmeas_B2	0	Cmeas_B2	0
12	Cmeas_C1	0	Cmeas_C1	0
13	Cmeas_C2	0	Cmeas_C2	0
14	Cmeas_A1	0	Cmeas_A1	0

APPENDIX D

****

AQUASIM Version 2.0 (win/mfc) - Parameter Estimation File

****

Number of parameters = 4
Number of data points = 10
Estimation method = secant

Parameters:

Name	Unit	Start	Minimum	Maximum
C_A1ini	mg/L	50.0003	0	1000
K_A	mg/L	564.73	0.01	1000
u_A	1/minutes	125.673	0.01	1000
Y_A		0.37786	0.01	10

Calculations:

C_A1ini [mg/L]	K_A [mg/L]	u_A [1/minutes]	Y_A []	Chi^2
50.0003	564.73	125.673	0.37786	37.7288
60.0003	564.73	125.673	0.37786	82.0774
50.0003	574.73	125.673	0.37786	37.747
50.0003	564.73	135.673	0.37786	38.0731
50.0003	564.73	125.673	0.47776	50.9204
50.0003	551.812	145.673	0.414361	38.0853
50.0003	550.015	148.455	0.450861	37.7537
55.0003	557.373	137.064	0.414361	48.7974
50.0003	535.301	112.096	0.338116	37.8988
52.5003	546.337	124.58	0.376239	40.5297
50.0002	623.588	125.644	0.328117	37.8636
51.2503	584.962	125.112	0.352178	38.4806
50.0003	564.73	125.673	0.37786	37.7288

Parameter estimation successfully finished (convergence criterion met)

C_A1ini [mg/L]	K_A [mg/L]	u_A [1/minutes]	Y_A []

Estimated values of the parameters:

50.0003 564.73 125.673 0.37786

Standard errors could not be estimated

Contribution of data series to Chi^2:

Calculation:	Data Series:	Chi^2 ini:	Chi^2 end:
fit1	Cmeas_A1	37.7288	37.7288
		37.7288	37.7288

Number of steps performed = 4
Number of simulations performed = 13

****

AQUASIM Version 2.0 (win/mfc) - Sensitivity Analysis File

****

Ranking of mean absolute sensitivities and error contributions:

Calculation Number: 1
Compartment: Reactor
Zone: Bulk Volume
Variable: C_A

	Parameter:	Sens AR: [mg/L]	Parameter:	Error Contr.: [mg/L]
1	C_A1ini	0.9484	Y_A	0.4687
2	Y_A	0.8855	C_A1ini	0.009484
3	u_A	0.8853	u_A	0.001409
4	K_A	0.8479	K_A	0.0003003
5	Cmeas_A1	0	Cmeas_A1	0
6	C_A2ini	0	C_A2ini	0
7	Cmeas_A2	0	Cmeas_A2	0
8	K_B	0	K_B	0
9	Cmeas_B	0	Cmeas_B	0
10	u_B	0	u_B	0
11	Cmeas_C	0	Cmeas_C	0
12	Y_B	0	Y_B	0

Variable: C_B

	Parameter:	Sens AR: [mg/L]	Parameter:	Error Contr.: [mg/L]
1	C_A1ini	26.59	Y_A	0.7141
2	u_B	14.31	Y_B	0.5609
3	Y_B	14.28	C_A1ini	0.2659
4	K_B	14.24	u_B	0.00581
5	u_A	1.353	u_A	0.002153
6	Y_A	1.349	K_A	0.0004587
7	K_A	1.295	K_B	0.000418
8	Cmeas_B	0	Cmeas_B	0
9	Cmeas_C	0	Cmeas_C	0
10	Cmeas_A1	0	Cmeas_A1	0
11	C_A2ini	0	C_A2ini	0
12	Cmeas_A2	0	Cmeas_A2	0

Variable: C_C

	Parameter:	Sens AR: [mg/L]	Parameter:	Error Contr.: [mg/L]
1	C_A1ini	22.47	Y_B	0.5609
2	u_B	14.31	Y_A	0.3478
3	Y_B	14.28	C_A1ini	0.2246
4	K_B	14.24	u_B	0.00581
5	u_A	0.6574	u_A	0.001046
6	Y_A	0.6572	K_B	0.000418
7	K_A	0.6295	K_A	0.0002229
8	Cmeas_B	0	Cmeas_B	0
9	Cmeas_C	0	Cmeas_C	0
10	Cmeas_A1	0	Cmeas_A1	0
11	C_A2ini	0	C_A2ini	0
12	Cmeas_A2	0	Cmeas_A2	0

APPENDIX E

****

AQUASIM Version 2.0 (win/mfc) - Parameter Estimation File

****

Number of parameters = 4
Number of data points = 10
Estimation method = secant

Parameters:

Name	Unit	Start	Minimum	Maximum
C_A1ini	mg/L	55.5708	0	1000
K_B	mg/L	9797.96	0.01	10000
u_B	1/minutes	675.216	0.01	1000
Y_B		10	0.01	100

Calculations:

C_A1ini [mg/L]	K_B [mg/L]	u_B [1/minutes]	Y_B []	Chi^2
55.5708	9797.96	675.216	10	21.6283
65.5708	9797.96	675.216	10	343.806
55.5708	9897.96	675.216	10	21.7612
55.5708	9797.96	685.216	10	21.8888
55.5708	9797.96	675.216	10.9999	32.3837
55.565	9780.18	655.217	9.70409	21.6321
55.5695	9597.96	681.846	10.3009	21.6282
60.5701	9697.96	678.531	10.1505	102.13
55.569	9197.96	695.105	10.9164	21.6481
58.0696	9447.96	686.818	10.5334	41.4486
55.5695	9597.96	681.846	10.3009	21.6282

Parameter estimation successfully finished (convergence criterion met)

C_A1ini [mg/L]	K_B [mg/L]	u_B [1/minutes]	Y_B []

Estimated values of the parameters:

55.5695 9597.96 681.846 10.3009

Standard errors could not be estimated

Contribution of data series to Chi^2:

Calculation:	Data Series:	Chi^2 ini:	Chi^2 end:
fit2	Cmeas_B	21.6283	21.6282
		---	---
		21.6283	21.6282

Number of steps performed = 3
Number of simulations performed = 11

****

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****

Ranking of mean absolute sensitivities and error contributions:

Calculation Number: 1
Compartment: Reactor
Zone: Bulk Volume

Variable: C_A

	Parameter:	Sens AR: [mg/L]	Parameter:	Error Contr.: [mg/L]
1	C_A1ini	3.127	Y_A	0.6943
2	Y_A	3.018	C_A1ini	0.02814
3	u_A	3.018	u_A	0.003672
4	K_A	2.936	K_A	0.0005873
5	Cmeas_A1	0	Cmeas_A1	0
6	C_A2ini	0	C_A2ini	0
7	Cmeas_A2	0	Cmeas_A2	0
8	K_B	0	K_B	0
9	Cmeas_B	0	Cmeas_B	0
10	u_B	0	u_B	0
11	Cmeas_C	0	Cmeas_C	0
12	Y_B	0	Y_B	0

Variable: C_B

	Parameter:	Sens AR: [mg/L]	Parameter:	Error Contr.: [mg/L]
1	C_A1ini	38.6	Y_A	0.8095
2	u_B	11.22	C_A1ini	0.3474
3	Y_B	11.2	Y_B	0.2175
4	K_B	11.17	u_A	0.004292
5	u_A	3.527	u_B	0.003291
6	Y_A	3.519	K_A	0.0006859
7	K_A	3.429	K_B	0.0002327
8	Cmeas_B	0	Cmeas_B	0
9	Cmeas_C	0	Cmeas_C	0
10	Cmeas_A1	0	Cmeas_A1	0
11	C_A2ini	0	C_A2ini	0
12	Cmeas_A2	0	Cmeas_A2	0

Variable: C_C

	Parameter:	Sens AR: [mg/L]	Parameter:	Error Contr.: [mg/L]
1	C_A1ini	13.84	Y_A	0.3167
2	u_B	11.22	Y_B	0.2175
3	Y_B	11.2	C_A1ini	0.1245

4	K_B	11.17	u_B	0.003291
5	u_A	1.378	u_A	0.001677
6	Y_A	1.377	K_A	0.000268
7	K_A	1.34	K_B	0.0002327
8	Cmeas_B	0	Cmeas_B	0
9	Cmeas_C	0	Cmeas_C	0
10	Cmeas_A1	0	Cmeas_A1	0
11	C_A2ini	0	C_A2ini	0
12	Cmeas_A2	0	Cmeas_A2	0