KINETICS OF BIODESULFURIZATION OF DIESEL AND KEROSENE

BY

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APRIL, 2010

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B.Tech (Hons) Chemistry, PGD, M. Eng.

(Chemical Engineering)

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SCHOOL OF POSTGRADUATE STUDIES
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This is to certify that the Thesis:

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By

KAREEM, SEMIU ADEBAYO
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DEDICATION

This work is dedicated to

Sekinat Oluwakemi

Shakiru Adekunmi

Sheriff Adedayo

&

Sadeeq Ademola

For their love, support and understanding

and above all The Almighty Allah (SWT)

ABSTRACT

The biodesulfurization of diesel and kerosene was investigated. Soil samples were taken at various points of the Ijora petroleum products haulage terminal, in Lagos, Nigeria. Microorganisms capable of selectively abstracting sulfur from diesel and kerosene without reducing their fuel values were isolated by using a sulfur-reducing selective medium. The choice of the sulfur selective reductive pathway is informed by the fact that it is sulfur specific and so the calorific value is maintained because C – C bonds are not altered in the pathway. The reaction pattern is similar to hydrodesulfurization (HDS) with respect to the production of H_2S. The H_2S that is formed in anaerobic route can be treated with existing refinery desulfurization plants (e.g. Claus process). The organisms were tentatively identified as *Desulfobacterium anilini* and *Desulfobacterium indolicum* based on their differential cultural, morphological and biochemical characteristics and by reference to *Bergey's Manual of Determinative Bacteriology*. The concentrations of various sulfur compounds were determined by Gas Chromatography with Pulsed Flamed Photometric Detector (GC-PFPD, type 5890A; Hewlett Packard, Mississauga, USA).

These microorganisms were found to desulfurize at least 70% of the fuels in 72 hours. Furthermore, *Desulfobacterium anilini* desulfurized the sulfur containing organic compounds in the fuels better than *Desulfobacterium indolicum* at the end of 72 hours for all the cases considered.

The rate expression for enzyme-catalyzed reactions was obtained by the method outlined by Boudart (1968) for the kinetics of heterogeneous catalysis. The rate expression obtained from the rate determining step has clearly shown that when the first step determines the rate (the formation of the enzyme-substrate complex from the reaction of the enzyme and substrate), the reaction is first order. On the other hand, the reaction follows the Michaelis-Menten kind of equation when the second step (conversion of the enzyme-substrate complex to the product) limits the rate of reaction.

Kinetic models were developed for both the sulfur specific oxidative biodesulfurization and sulfur specific reductive biodesulfurization pathways. The development of the sulfur specific oxidative biodesulfurization pathway was based on the 4S pathway of biodesulfurization, a multiple step single substrate system, while the kinetic model of sulfur specific reductive biodesulfurization was developed based on the pathway developed by Kim *et al.* (1985). The pathway is for a single step, single substrate system. The obtained kinetic expressions are hyperbolic. Furthermore, the influence of mass transfer on the kinetics of biodesulfurization was also investigated. The kinetic parameters, the maximum rate constant k and the Michaelis-Menten constant K_M, obtained from linear plots such as Eadie-Hofstee, Hanes and Lineweaver-Buck plots were subjected to Macquardt's non-linear regression analysis before they were used in simulating the biodesulfurization. The partition (distribution) coefficient used in the simulation on the other hand was estimated through the efficacy of combining linear solvation energy relationships (LSERs) developed for pure systems through the application of linear solvent strength theory. The developed models were solved numerically by the Finite Difference method. The simulation results showed very good agreement with the experimental results and that the kinetics of sulfur selective reductive biodesulfurization is influenced by mass transfer.

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LIST OF SYMBOLS

(A) = Concentration of reactant component in an enzyme-catalyzed reaction

A = Cross sectional area of a reactor (m^2)

a = Ratio of surface area to the volume of a catalyst (m^2/m^3)

a_i = Pseudo rate constant of reaction(s^{-1})

$A_{i,j}$ = Elements of a matrix

C = Substrate concentration (mg/L)

C_S = Concentration of the substrate on the surface of enzyme (mg/L)

d = Dimension

E = Enzyme

E = vector of difference between experimental and predicted molar quantities

E1 = DBT monooxygenase, an enzyme

E2 = DBT sulfone monooxygenase, an enzyme

E3 = Hydroxybiphenyl desulfinase, an enzyme

ES = Enzyme-substrate complex

F = Molar quantity (mole/unit volume)

F_i = Predicted molar quantity (mole/unit volume)

F_i^0 = Experimental molar quantity (mole/unit volume)

g = arbitrary value suitable for iteration

H = Hydrogen radical

I = Identity matrix

K = Distribution coefficient

k = maximum rate constant (mg/L.hr)

Kb = kilobase

k_{-i} = Backward rate constant at ith stage of equilibrium reaction

k_i = Forward rate constant at ith stage of equilibrium reaction

K_M = Michaelis-Menten constant (mg/L)

k_m = kinetic parameters

L = Number of experiments

L = Total concentration of active sites on the enzyme surface

N = Notation

N = Number of components in an experiment

n = order of reaction

N, D = Determinants in a matrix

P = Product (mg/L)

P1 = DBT sulfoxide

P2 = DBT sulfone

P3 = 2-Hydroxylbiphenyl-2-sulfinic acid

P4 = 2-Hydroxylbiphenyl

P5 = Biphenyl

pth, qth= Order in space

q = Amount of substrate adsorbed on the surface of biocatalyst (mg/L)

r = rate of reaction (moles/volume of reactor-time)

r_{ac} = rate of accumulation

r_i = Possible locations where mass transfer could be encountered

r_m = rate of mass transfer

S = Substrate

t = Time (s)

T = Temperature (K)

u = velocity component of a reaction (ms^{-1})

v = Velocity (ms^{-1})

V = Volume of a reactor (m^3)

Z = Length of a reactor (m)

GREEK LETTERS

δ = Parametric correction factor

ϕ = Sum of squares of the residuals

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Oil refineries are facing many challenges, including use of heavier crude oils, increased fuel quality standards, and a need to reduce air pollution emissions (Mercelis, 2002). It is a known fact that the combustion of fossil fuels gives rise to the formation of atmospheric gaseous pollutants which are harmful to human, animal and plant life. For example, Carbon (IV) oxide emissions have been implicated in global warming. Secondly, nitrogen and sulfur oxides emissions have been shown to be responsible for acid rain, which leads to destruction of buildings, deforestation and lake water poisoning (Mederos *et al.,* 2006). Governments throughout the world have recognized the problems associated with these emissions and have moved to reduce them through legislations. Regulations for the sulfur level in diesel oil have become increasingly stringent, thus global society is stepping on the road to zero-sulfur fuel, with only differences in the starting point of sulfur level and rate reduction of sulfur content between different countries (Constanti *et al.,* 1994).

To meet regulated sulfur levels, petroleum fuels must be treated to remove organic sulfur. Oil-refining industries have to cope with more stringent specifications on the sulfur content that are driven by environmental concerns (Anabtawi *et al.,* 1996). Hydrodesulfurization (HDS) is the most common technology used by refineries to remove sulfur from intermediate streams (Speight, 1980). HDS is a catalytic process that converts organic sulfur to hydrogen sulfide gas by reacting crude oil fractions with hydrogen at pressures between 1 and 20 MPa and temperatures between 290 and 455 ^{o}C, depending on the feed and level of desulfurization required (Oyekunle, 2004).

In general, crude oil once refined, yields three basic groupings of products that are produced when it is broken down into cuts or fractions, namely:

- The Gas or Gasoline fractions form the lower boiling products. They include Liquefied Petroleum Gas (LPG), naphtha, aviation fuel, motor fuel and feed stocks for the petrochemical industry.
- The Middle Distillates refer to products from the middle boiling range of petroleum and include kerosene, diesel fuel, distillate fuel oil and light gas oil; waxy

distillate and lower boiling lubricating oils are sometimes included in the middle distillates.

- The remainder of the crude oil includes the higher boiling lubricating oils, gas oils and residuum (the non-volatile fraction of the crude oil). Residuum can produce heavy lubricating oils and waxes, although it is often used for asphalt production.

Organic sulfur compounds in the lower-boiling fractions of petroleum, such as, the gasoline range, are mainly thiols, sulfides and thiophenes, which are readily removed by HDS (Wang and Kraweic, 1994). However, middle-distillate fractions, like diesel, kerosene and some fuel oil range, contain significant amounts of benzothiophenes and dibenzothiophenes (DBTs), which are considerably more difficult to remove by HDS. Among the most refractory of these compounds are DBTs with substitutions adjacent to the sulfur moiety. Compounds of this type are said to be sterically hindered because the substitutions are believed to sterically hinder access of the sulfur atom to the catalyst surface due to their resistance to HDS. Consequently, sterically hindered compounds represent a significant barrier to reaching very low sulfur levels in middle- and heavy-distillate-range fuels. The high cost and inherent chemical limitations associated with HDS make alternatives to this technology to be of interest to the petroleum industry. Moreover, current trends toward stricter regulations on the content of sulfur in fuels provide incentive for the continued search for improved desulfurization processes. The hydrogen sulfide produced as a result of HDS is a corrosive gaseous substance. Elevated or persistent levels of hydrogen sulfide are known to poison (inactivate) the HDS catalyst, thereby complicating the desulfurization of petroleum crude and products that are high in sulfur (Chang *et al.,* 1998).

Organic sulfur in petroleum fossil fuels is present in a myriad of compounds, some of which are unstable in that they cannot readily be desulfurized or refractory because they do not easily yield to conventional desulfurization treatment by HDS. Increasing the severity of HDS also elicits undesirable effects on fuel quality as other chemical components are reduced at the higher temperatures and pressures needed to achieve low sulfur levels (Egorova, 2003).

In middle distillate (diesel range) fractions, the sulfur that remains after aggressive HDS treatment is typically in the form of dibenzothiophene (DBT) and its substituents compounds. The most refractory DBTs have substituents at the 4 and 6 positions, which are adjacent to the sulfur moiety and are believed to sterically hinder access of the sulfur atom to the catalyst surface (Kabe *et al.,* 1992). As regulations on sulfur levels in fuels become stricter, more of the HDS-refractory compounds must be removed. As a result, HDS-refractory sulfur compounds represent a significant barrier to reaching very low sulfur levels in the middle and heavy distillate range fuels (Marcelis, 2002).

Biodesulfurization is a process in which sulfur is removed by a bio-catalyst yielding sulfate or sulfide as a product. Early work on biodesulfurization focused on organisms that degrade DBT. The pathways involved relied on oxidation and mineralization of the DBT carbon skeleton instead of sulfur removal and thus reduced the fuel value of the desulfurized product (Kodama *et al*, 1970 and 1973). This pathway is known as the Kodama pathway of degradation of DBT.

Recent studies focused on organisms that use sulfur-selective oxidative and reductive pathways to remove sulfur from organic sulfur compounds and are capable of desulfurizing DBT and sterically hindered DBT compounds (Lee *et al.,* 1995). A number of bacteria that use the sulfur-selective oxidative desulfurization pathway have been isolated (Campbell, 1993 and Grossman, 1996). This pathway referred to as the 4S pathway involves sequential oxidation of the sulfur moiety and cleavage of the carbon – sulfur bonds. The sulfur-selective reductive desulfurization pathway is one in which the sulfur-containing organic content of the fuel serves as electron acceptor and subsequent removal of the sulfur.

The genes encoding DBT desulfurization have been extensively characterized and have been named Sox (sulfur oxidation) by Denome *et al.,* (1994) and Dsz (desulfurization) by Piddington *et al.,* (1995). Both groups identified three genes,

forming a single operon, which was necessary and sufficient to confer the desulfurization activity to the microorganism.

Previous work on sulfur selective pathway has focused on model compounds most especially DBT. Scanty work has been reported on the biodesulfurization of real refinery feeds and some petroleum products (Fang *et al.,* 2006) limiting the ability to assess the commercial potential of biodesulfurization. In this work, kinetic models of biodesulfurization of diesel and kerosene were developed based on the sulfur specific reductive pathway (Campbell, 1993 and Grossman, 1996); the mass transfer effect was also investigated and subsequently coupled with the developed kinetics. Experimental study of the biodesulfurization of diesel and kerosene was conducted using microorganisms isolated from petroleum products-polluted soil in Lagos, Nigeria.

1.2 STATEMENT OF THE PROBLEM

Oil-refining industries have to cope with more stringent specifications on the sulfur contents that are driven by environmental concerns (Anabtawi *et al.,* 1996). During the last decade, clean air considerations have led to drastic reductions in the allowable sulfur content for diesel. The stricter regulations on the sulfur content for diesel are an impetus to perform research on deep desulfurization to obtain gas oils with low sulfur concentrations. To give an outline: 75% of the refractory sulfur compounds must be converted, without altering the remaining hydrocarbons that constitute more than 98% of the diesel (Segawa *et al.,* 2000). The use of insufficiently desulfurized distillates as fuels results in the formation of sulfur oxides. These sulfur-containing emissions contribute to the acid deposition (‘acid rain’) and were the impetus for stringent legislation. Future restrictions on the sulfur content are mainly driven to reduce the amount of particulates formed during the burning of the fuel and to improve the applicability of exhaust catalysis. Apart from legislative constraints, downstream catalytic requirements have an impact on the allowable sulfur content in oil fractions, *e.g.* catalytic reforming processes are more efficient in the absence of

organic sulfur compounds. Unfortunately, there is a trend towards higher sulfur contents of the crude oil reserves. Easily accessible and relatively low sulfur oil-reserves are being depleted; consequently reserves with higher sulfur contents must be used as feedstock for refining processes.

In this study the potential of biodesulfurization in the deep removal of sulfur from diesel and kerosene were investigated. It was also intended to isolate and identify microorganisms that can desulfurize diesel and kerosene.

1.3 PURPOSE OF THE STUDY

Commercial fossil fuels in the middle-distillate fraction of petroleum such as diesel and kerosene contain heteroatoms such as nitrogen, oxygen and sulfur. The sulfur-containing substances on combustion give rise to oxides of sulfur. The sulfur oxides are well known precursors for acid rain with adverse consequence in aquatic and soil ecosystems.

Against this background, the purpose of this research study was to investigate a method that could lead to the development of low processing and design costs for the biodesulfurization of diesel and kerosene as an alternative or complimentary process to hydrodesulfurization.

The objectives of this research study are therefore:

♦ To investigate the desulfurization of selected petroleum products, namely diesel and kerosene.

♦ To isolate microorganisms capable of biodesulfurizing diesel and kerosene and investigate their influence on these substrates.

♦ To develop a kinetic model for the biodesulfurization of diesel and kerosene and provide simulation of substrate concentration versus time profile.

1.4 RESEARCH QUESTIONS

The following research questions will guide this research work:

♦ What are the current desulfurization techniques and their shortcomings in terms of effectiveness in meeting stringent environmental regulations?

♦ How does this present research study differ from the work of previous researchers?

♦ Would the developed models be universally applicable to all biodesulfurization reactions?

1.5 THEORETICAL FRAMEWORK OF STUDY

This research work focuses on the development of suitable models for the biodesulfurization of diesel and kerosene based on the following theoretical concepts:

(i) The thorough understanding of the mechanism of the biodesulfurization reaction.

(ii) The development of kinetic rate expressions for biodesulfurization based on sulfur selective pathway of biodesulfurization.

(iii) The use of the developed kinetic expressions in conjunction with mass transport of the substrates onto the surface of the organism.

(iv) The selective removal of the sulfur from the substrates without distorting significantly their carbon framework by extracellular enzymes activities of the microorganisms.

(v) The use of finite difference scheme to solve the resulting model which is a first order differential equation.

1.6 SIGNIFICANCE OF THE STUDY

1. The potential of biodesulfurization as a means of effectively meeting the stringent requirements of the various environmental regulatory agencies is receiving wide acceptability all over the world.
2. The method depends on a process that would make the substrate available to microorganisms that could effectively and efficiently biodesulfurize the fuel. Two microorganisms, *Desulfobacterium indolicum* and *Desulfobacterium anilini* have been isolated and found to desulfurize diesel and kerosene very well.
3. A mass transfer dependent kinetic analysis of biodesulfurization was developed and the outcome was used to simulate the experimental results.
4. The quantitative parameters (measured, calculated and estimated) represent a valuable pool of information that can be used for reactor design that will take the technology to the market place.
5. Microbial metabolism of organosulfur compounds is of interest in the petroleum industry not only for the biodesulfurization but also for their in-situ reduction of viscosity of the oil.
6. It presents opportunities for the biocatalytic production of specialty chemicals which include sulfinates, a starting material for novel surfactants.
7. It also provides a clean-up technology for the effluents from textile industries which are known to contain high levels of organosulfur compounds.
8. This study which involves the biodesulfurization of diesel and kerosene is a complete departure from the usual practice of studying model substrates.

1.7 LIMITATIONS AND SCOPE OF STUDY

This research work is limited to the development of a protocol, for evaluating biodesulfurization in middle distillate petroleum fractions. It is covered within the scope of:

1. Detailed and comprehensive literature review of biodesulfurization

2. Model development for the kinetics of the mechanism of biodesulfurization
3. Investigating the influence of mass transfer on biodesulfurization
4. Isolation of microorganisms capable of biodesulfurization
5. Obtaining experimental results from the action of the isolated microorganisms on diesel and kerosene
6. Numerical simulation of biodesulfurization parameters
7. Testing and validation of theoretical models with data from experiments

1.8 OPERATIONAL DEFINITION OF TERMS

Adsorption: This is a process that occurs when solute accumulates on the surface of a solid, forming a film of atoms or molecules.

Anoxic conditions: Anoxic conditions will occur when the rate of oxidation of organic matter by bacteria is greater than the supply of dissolved oxygen.

API Gravity: This is the American Petroleum Institute's inverted scale for denoting the lightness or heaviness of crude oils and other liquid hydrocarbon. The lighter the crude, the heavier the API gravity.

Basic Compounds: In chemistry, a base is most commonly thought of as an aqueous substance that can accept hydrogen ions. A base is also often referred to as an alkali if OH^- ions are involved.

Bioavailability: This is a term used by several branches of scientific study to describe the way chemicals are absorbed by humans and other animals**.** Bioavailability was once strictly ascribed to pharmacolog, but now has broad applications in environmental sciences as well. Bioavailability, when used in environmental sciences, evaluates the rate and amount of toxic substances that may occur in the body.

Biodesulfurization: This is the biological removal of sulfur from a system by microorganisms.

Bubble-Train Flow: Bubble-train flow consists of strings of bubbles separated by liquid slugs flowing concurrently inside capillaries of circular or square cross section. Bubble-train flows have interesting properties and have been used to enhance heat and mass transfer rates inside capillaries.

Buffer Solution: This is an aqueous solution consisting of a mixture of weak acid and its conjugate base or a weak base and its conjugate acid.

Cell Surface Hydrophobicity: This is a very important physicochemical feature of a microorganism which has a great influence in the ability of such organism to adhere

to the surface of a host. The host could be cells or other substances like hydrocarbon.

Chromatography: This is the collective term for a family of laboratory techniques for the separation of mixtures. It involves pairing a mixture dissolved in a mobile phase through a stationary phase which separates the analytes to be measured from the other molecules in the mixture and allows it to be isolated.

Chromatography Detector: This is a device that locates in the dimension of space and time, the position of the components of a mixture that has been subjected to a chromatographic process and thus permits the senses to appreciate the nature of the separation.

Concurrent Flow: In system where this occurs, the two fluids flow in the same direction.

Countercurrent Flow: When two fluid flow in opposite directions, the system can maintain a nearly constant gradient between the two flows over entire length.

Cytochromes are, in general, membrane-bound hemoproteins that contain heme groups and carryout electron transport. They are found either as monomeric proteins (e.g. cytochrome c) or as subunits of bigger enzymatic complexes that catalyze redox reactions.

DBT monooxygenases, DszC: This is the enzyme responsible for the first two oxidations in the sulfur specific pathway of biodesulfurization.
DBT sulfone P_2: This is the second intermediate product in the sulfur specific pathway of biodesulfurization.

DBT sulfone monooxygenases, DszA: This is the enzyme responsible for the first cleavage of the C-S bond in the sulfur specific pathway of biodesulfurization.

DBT sulfoxide, P_1: This is the first intermediate product in the sulfur specific pathway of biodesulfurization.

Directed Evolution is a method used in protein engineering to harness the power of natural selection to evolve proteins or RNA with desirable properties not found in nature.

Distribution coefficient, K: This is the ratio of concentrations of a compound in two phases of a mixture of two immiscible solvents at equilibrium. Hence, it is a measure of differential solubility of the compound between these two solvent.

Elution: This is a term used in analytical chemistry to describe the emergence of materials with specific chemical composition from the column of a chromatograph.

Enrichment Culture: This is a medium with specific and known qualities that favors the growth of a particular microorganism. The enrichment culture's environment will support the growth of a selected microorganism while inhibiting the growth of others.

Enzymes, E: These are biomolecules that catalyze chemical reactions.

Fischer-Tropsch Process: This is also sometimes called Fischer-Tropsch synthesis is a catalyzed chemical reaction in which synthesis gas, a mixture of carbon monoxide and hydrogen, is converted into liquid hydrocarbon of various forms.

Flammable liquid: Generally, a flammable liquid means a liquid which may catch fire easily. In the USA, there is a precise definition of flammable liquid as one with a flashpoint below 38^0C. Less-flammable liquids (with a flashpoint between 38^0C and 93^0C) are defined as combustible liquids.

Fouling refers to the accumulation of unwanted material on solid surfaces, most often in an aquatic environment. The fouling material can consist of either living organisms (bio-fouling) or a non-living substance (inorganic or organic).

Gas-Liquid Chromatography: It is commonly referred to as simply GC is a common type of chromatography used for separating and analyzing compounds that can be separated without decomposition.

Genotype: Is the genetic constitution of a cell, an organism, or an individual (i.e. the specific allele makeup of the individual) usually with reference to a specific character under consideration.

Greenhouse Gases: These are gases in an atmosphere that absorb and emit radiation within the thermal infrared range.

Gross Domestic product, GDP: Also called the gross domestic income (GDI) is a basic measure of an economy's economic performance. It is the market value of all final goods and services made within the borders of a nation in a year.

Heterocyclic Compounds: These are organic compounds containing at least one atom of carbon, and at least another other than carbon such as sulfur, oxygen or nitrogen within a ring structure.

Heterogeneous Catalysis: This is a chemistry term which describes catalysis where the catalyst is in a different phase from the reactants.

Hydrogenation is a chemical reaction where hydrogen is added to a molecule without cleaving bonds.

Hydrogenolysis: This occurs when carbon- carbon or carbon-heteroatom single bond is cleaved catalytically using hydrogen.gas.

Hydrophilic: This refers to a physical property of a molecule that can transiently bond with water through hydrogen bonding.

Hydrophobicity: Is the property exhibited by non-polar molecules by tending to form aggregates of like molecules in water and analogous intramolecular interactions.

Hydroxybiphenyl desulfinase, DszB: This is the enzyme responsible for the production of sulfite and an intact hydrocarbon molecule, which is the last reaction of the sulfur specific pathway of biodesulfurization.

Immobilized Cell: Is an alternative system to enzyme immobilization. Unlike enzyme immobilization, where enzyme is attached to a substrate (such as calcium alginate), in an immobilized cell systems, the target cell is immobilized. Such methods may be implemented when the enzymes required are difficult or expensive to extract.

Isolation: This is a commonly applied method of obtaining or extracting a pure culture of organisms developing in an enrichment culture.

Karrick Process: Is a low-temperature carbonization (LTC) and pyrolysis process of carbonaceous materials. Although primarily meant for coal carbonization, it could also be used for processing of oil shale, lignite or any carbonaceous material.

Maximum rate constant, k: This is defined as the maximum rate attainable in the course of a reaction. Its unit is dependent upon the order of reaction.
Michaelis-Menten constant, K_M: This is the substrate concentration when the reaction rate is half its maximum value.

Mineralization: This is the process of converting an organic substance to inorganic forms.

Mobile Phase: This is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen.

NADH-flavin mononucleotide oxidoreductase, DszD: This is the enzyme that supplies the two monooxygenases with reduced flavin.

Non-Linear regression analysis: This is a form of regression analysis in which observational data are simulated by a function which is a non-linear combination of model parameters and depends on one or more independent variable.

Order of Reaction: Order of reaction in chemical kinetics, with respect to certain reactants, is defined as the power to which its concentration term in the rate equation is raised. However, it can only be determined by experiments.

Phenotype: Any observable characteristic or trait of an organism such as its morphology, development, biochemical or physiological properties, or behavior. Phenotypes result from the expression of an organism's genes as well as the influence of environmental factors and possible interactions between the two.

Plasmid: An extra-chromosomal DNA molecule separate from the chromosomal DNA which is capable of replicating independently of the chromosomal DNA. In many cases, it is circular and double stranded. Plasmids usually occur naturally in bacteria.

Perturbation Theory: This comprises mathematical methods that are used to find an approximate solution to a problem which cannot be solved exactly.

Precursor: A precursor is a compound that participates in chemical reaction that produces another compound.

Rate of substrate consumption, r: This is the amount of substrate catalyzed per unit time in the course of a reaction. It is normally measured in terms of mol/unit time.

Refractory: Resistant to treatment

Retention time: This is the period which the compound being analyzed interact with walls of the column, which are coated with different stationary phases. The comparison of retention times is what gives GC its analytical usefulness.

Stationary phase: This is a microscopic layer of liquid or polymer on an inert solid support inside a piece of glass or metal tube called a column.

Steric Hindrance: This occurs when the size of groups within a molecule prevents chemical reactions that are observed in related smaller molecules. It can be a problem sometimes, it can also be a very useful tool often exploited by chemists to change the reactivity pattern of a molecule by stopping unwanted side-reactions.

Surfactants: They are wetting agents that lower the surface tension of a liquid, allowing easier spreading, and lower the interfacial tension between two liquids.

Thermal Depolymerization (TDP): It is a process using hydrous pyrolysis for the reduction of complex organic material (usually waste products of various sorts,often known as biomass and plastic) into light crude oil.

Thermocline (Metalimnion): Is a thin but distinct layer in a large body of fluid (e.g. water, such as ocean or lake, or air such as an atmosphere), in which temperature changes more rapidly with depth than it does in the layers above or below.

Transposons: These are sequences of DNA that can move around to different positions within the genome of a single cell, a process called transposition. In the process, they can cause mutation and change the amount of DNA in the genome.

Ultrasound: This is a cyclic sound pressure with a frequency greater than the upper limit of human hearing. It is approximately 20 KHz in healthy young adult and thus serves as a useful lower limit in describing ultrasound.

Viscosity: This is a measure of the resistance of a fluid which is being deformed by either shear stress or extensional stress. In everyday terms (and for fluids only), viscosity is “thickness”

2-hydroxybiphenyl-2’-sulfinic acid, P_3: This is the third intermediate product in the sulfur specific pathway of biodesulfurization.

2-hydroxybiphenyl, P: The intact hydrocarbon molecule, final product in the sulfur specific pathway of biodesulfurization.

CHAPTER 2

LITERATURE REVIEW

2.1 PETROLEUM FORMATION, COMPOSITION AND DISTRIBUTION

After food, fossil fuel is humanity's most important source of energy. Many of the benefits we enjoy from our way of life are due to fossil fuel use. Most of our energy (about 85%) comes from fossil fuel; another 8% comes from nuclear power and 7% from all other sources, mostly hydroelectric power and wood. There are three major fossil fuels — coal, oil, and natural gas. Oil leads with a share of about 40% of the total world consumption, followed by coal (24%) and natural gas (22%). Almost all fossil fuels are used by burning, which causes pollution. It produces waste products due to impurities in the fuel, especially particulates and various gases such as sulfur dioxide, nitrogen oxides and volatile organic compounds (Alves *et al*., 2005).

Petroleum or crude oil is a naturally occurring, flammable liquid found in rock formations in the earth consisting of a complex mixture of hydrocarbons of various molecular weights, plus other organic compounds. The proportion of hydrocarbons in the mixture is highly variable and ranges from as much as 97% by weight in the lighter oils to as little as 50% in the heavier oils and bitumens (Arenskotter *et al*., 2004).

Crude oil varies greatly in appearance depending on its composition. It is usually black or dark brown (although it may be yellowish or even greenish). In the reservoir it is usually found in association with natural gas, which, being lighter, forms a gas cap over the petroleum, and saline water, which being heavier generally floats underneath it. Crude oil may also be found in semi-solid form mixed with sand, as in the Athabasca oil sands, Canada, where it may be referred to as crude bitumen (Caro *et al*., 2007).

The hydrocarbons in crude oil are mostly alkanes, cycloalkanes, and various aromatic hydrocarbons while the other organic compounds contain nitrogen, oxygen, and sulfur along with trace amounts of metals such as iron, nickel, copper and vanadium. The exact molecular composition varies widely from formation to formation

but the proportion of chemical elements varies over fairly narrow limits as shown in Table 2.1(Speight, 1999*):*

Table 2.1: Proportion of Chemical Elements in Crude Oil

Content	**% Composition**
Carbon	83 – 87
Hydrogen	10 – 14
Nitrogen	0.1 – 2
Oxygen	0.1 – 1.5
Sulfur	0.5 – 6
Metals	< 0.01

Petroleum is used mostly, by volume, for producing fuel oil and gasoline (petrol) and both are important "primary energy" sources (IEA Key World Energy Statistics). Thus, about 84% by volume of the hydrocarbons present in petroleum is converted into energy-rich fuels (petroleum-based fuels), including gasoline, diesel, jet, heating, and other fuel oils and liquefied petroleum gas ("Crude oil is made into different fuels") (Fang *et al*., 2006). Due to its high energy density, easy transportability and relative abundance, it has become the world's most important source of energy since the mid-1950s. Petroleum is also the raw material for many chemical products, including pharmaceuticals, solvents, fertilizers, pesticides and plastics; the 16% not used for energy production is converted into these other materials (Jia *et al*., 2006).

Petroleum is found in porous rock formation in the upper strata of some areas of the earth's crust. There is also petroleum in oil sands (tar sands). Globally known reserves of petroleum are typically estimated at around 140 km^3 (1.2 trillion barrels) without oil sands (EIA reserves estimates), or 440 km^3 (3.74 trillion barrels) with oil sands (CERA report on total world oil). However, oil production from oil sands is currently severely limited. Consumption is currently around 84 million barrels per day, or 3.6 km^3 per year. The energy return over energy invested (EROEI) ratio of oil is constantly falling as petroleum recovery gets more difficult, recoverable oil reserves

are significantly less than total oil-in-place. At current consumption levels, and assuming that oil will be consumed only from reservoirs, known recoverable reserves would be gone around 2039, potentially leading to a global energy crisis. However, there are factors which may extend or reduce this estimate, including the rapidly increasing demand for petroleum in China, India, and other developing nations; new discoveries; energy conservation and use of alternative energy sources; and new economically viable exploitation of non-conventional oil sources (Marcelis, 2002).

Petroleum is a mixture of a very large number of different hydrocarbons; the most commonly found molecules are alkanes (linear or branched), cycloalkanes, aromatic hydrocarbons, or more complicated chemicals like asphaltenes. Each petroleum variety has a unique mix of molecules, which define its physical and chemical properties, like color and viscosity.

The alkanes, also known as paraffins, are saturated hydrocarbons with straight or branched chains which contain only carbon and hydrogen and have the general molecular formula C_nH_{2n+2} They generally have from 5 to 40 carbon atoms per molecule, although trace amounts of shorter or longer molecules may be present in the mixture. The alkanes from pentane (C_5H_{12}) to octane (C_8H_{18}) are refined into gasoline (petrol), the ones from nonane (C_9H_{20}) to hexadecane ($C_{16}H_{34}$) into diesel fuel and kerosene (primary component of many types of jet fuel), and the ones from hexadecane upwards into fuel oil and lubricating oil. At the heavier end of the range, paraffin wax is an alkane with approximately 25 carbon atoms, while asphalt has 35 and up, although these are usually cracked by modern refineries into more valuable products. Any shorter hydrocarbons are considered natural gas or natural gas liquids (Maghsoudi *et al*., 2001).

The cycloalkanes, also known as napthenes, are saturated hydrocarbons which have one or more carbon rings to which hydrogen atoms are attached according to the formula C_nH_{2n}. Cycloalkanes have similar properties to alkanes but have higher boiling points. The aromatic hydrocarbons are unsaturated hydrocarbons which have one or more planar six-carbon rings called benzene rings, to which hydrogen atoms

are attached with the formula C_nH_n. They tend to burn with a sooty flame, and many have a sweet aroma. Some are carcinogenic.

These different molecules are separated by fractional distillation at an oil refinery to produce gasoline, jet fuel, kerosene, and other hydrocarbon-based products. Incomplete combustion of petroleum or gasoline results in production of potentially toxic by-products. Too little oxygen results in Carbon (II) oxide. Due to high temperatures and high pressures involved, exhaust gases from gasoline combustion in car engines usually include nitrogen oxides which are responsible for creation of photochemical smog (Nomura *et al*., 2005).

2.2 CLASSIFICATION

The petroleum industry classifies "crude" by the location of its origin (e.g., "West Texas Intermediate, WTI" or "Brent") and often by its relative weight or viscosity ("light", "intermediate" or "heavy");, Refiners may also refer to it as "sweet," which means it contains relatively little sulfur, or as "sour," which means it contains substantial amounts of sulfur and requires more refining in order to meet current product specifications. Each crude oil has unique molecular characteristics which are understood by the use of crude oil assay analysis in petroleum laboratories.

Barrels from an area in which the crude oil's molecular characteristics have been determined and the oil has been classified are used as pricing references throughout the world. These references are known as Crude oil benchmarks:

- Brent Crude, comprising 15 oils from fields in the Brent and Ninian systems in the East Shetland Basin of the North Sea. The oil is landed at Sullom Voe terminal in the Shetlands. Oil production from Europe, Africa and Middle Eastern oil flowing West tends to be priced off the price of this oil, which forms a benchmark.
- West Texas Intermediate (WTI) for North American oil.
- Dubai, used as benchmark for Middle East oil flowing to the Asia-Pacific region.
- Tapis (from Malaysia, used as a reference for light Far East oil)
- Minas (from Indonesia, used as a reference for heavy Far East oil)

- The OPEC Reference Basket, a weighted average of oil blends from various OPEC (The Organization of the Petroleum Exporting Countries) countries.

2.3 ACTIVITIES IN THE PETROLEUM INDUSTRY

The main activities of the petroleum industry are:

- Exploration
- Production
- Processing (refining)
- Marketing and distribution

The most common method of obtaining petroleum is extracting it from oil wells found in oil fields. With improved technologies and higher demand for hydrocarbons various methods are applied in petroleum exploration and development to optimize the recovery of oil and gas (Enhanced Oil Recovery, EOR). Primary recovery methods are used to extract oil that is brought to the surface by underground pressure, and can generally recover about 20% of the oil present. The natural pressure can come from several different sources; where it is provided by an underlying water layer and is called a water-driven reservoir but where it is from the gas cap above, it is called gas-driven. When the reservoir pressure is depleted to the point that the oil is no longer brought to the surface, secondary recovery methods are employed to draw another 5 to 10% of the oil in the well to the surface. In a water-driven oil field, water can be injected into the water layer below the oil, and in a gas-driven field it can be injected into the gas cap above to repressurize the reservoir. Finally, when secondary oil recovery methods are no longer viable, tertiary recovery methods reduce the viscosity of the oil in order to bring more to the surface. These may involve the injection of heat, vapor, surfactants, solvents, or miscible gases as in Carbon(IV)oxide flooding (Percheco *et al*., 1999).

During the oil price increases of 2004-2008, alternative methods of producing oil gained importance. The most widely known alternatives involve extracting oil from sources such as oil shale or tar sands. These resources exist in large quantities;

however, extracting the oil at low cost without excessively harming the environment remains a challenge.

It is also possible to chemically transform methane or coal into the various hydrocarbons found in oil. The best-known method is the Fischer-Tropsch process. It was a concept pioneered in Nazi Germany when imports of petroleum were restricted due to war and Germany found a method to extract oil from coal. It was known as Ersatz (English:"substitute") oil, and accounted for nearly half the total oil used in the second world war by Germany. However, the process was used only as a last resort as naturally occurring oil was much cheaper. As crude oil prices increase, the cost of coal to oil conversion became comparatively cheaper. The method involves converting high ash coal into synthetic oil in a multi-stage process (Speight, 1980).

Currently, two companies have commercialized their Fischer-Tropsch technology. Shell Oil in Bintulu, Malaysia, uses natural gas as a feedstock, and produces primarily low-sulfur diesel fuels (Shell Middle Distillate Synthesis Malaysia) and Sasol in South Africa uses coal as a feedstock, and produces a variety of synthetic petroleum products. The process is today used in South Africa to produce most of the country's diesel fuel from coal by Sasol. The process was used in South Africa to meet its energy needs during its isolation under Apartheid. This process produces low sulfur diesel fuel but also produces large amounts of greenhouse gases.

An alternative method of converting coal into petroleum is the Karrick process, which was pioneered in the 1930s in the United States. It uses high temperatures in the absence of ambient air, to distill the short-chain hydrocarbons out of coal instead of petroleum.

More recently explored is thermal depolymerization (TDP), a process for the reduction of complex organic materials into light crude oil. Using pressure and heat, long chain polymers of hydrogen, oxygen and carbon decompose into short-chain hydrocarbons. This mimics the natural geological processes thought to be involved in

the production of fossil fuels. In theory, thermal depolymerization can convert any organic waste into petroleum substitutes (Monticello, 2000).

The first commercial oil well drilled in North America was in Oil Springs, Ontario, Canada in 1858, dug by James Miller Williams. The US petroleum industry began with Edwin Drake's drilling of a 69-foot (21 m) oil well in 1859, on Oil Creek near Titusville, Pennsylvania, for the Seneca Oil Company (originally yielding 25 barrels per day, by the end of the year output was at the rate of 15 barrels per day). The industry grew slowly in the 1800s, driven by the demand for kerosene and oil lamps. It became a major national concern in the early part of the 20th century; the introduction of the internal combustion engine provided a demand that has largely sustained the industry to this day. Early "local" finds like those in Pennsylvania and Ontario were quickly outpaced by demand, leading to "oil booms" in Texas, Oklahoma, and California. By 1910, significant oil fields had been discovered in Canada (specifically, in the province of Ontario), the Dutch East Indies (1885, in Sumatra), Iran (1908, in Masjed Soleiman), Peru, Venezuela, and Mexico, and were being developed at an industrial level (Monticello, 2000).

Until the mid-1950s, coal was still the world's foremost fuel, but oil quickly became the leading fuel. Following the 1973 and 1979 energy crisis, there was significant media coverage of oil supply levels. This brought to light the concern that oil is a limited resource that will eventually run out, at least as an economically viable energy source. At the time, the most common and popular predictions were quite dire. However, a period of increase production and reduced demand caused an oil glut in the 1980s (Van Hamme *et al*., 2003).

Today, about 90% of vehicular fuel needs are met by oil. Petroleum also makes up 40% of total energy consumption in the United States, but is responsible for only 2% of electricity generation. Petroleum's worth as a portable, dense energy source powering the vast majority of vehicles and as the base of many industrial chemicals makes it one of the world's most important commodities. Access to it was a major factor in several military conflicts including World War II and the Persian Gulf Wars of

the late twentieth and early twenty-first centuries. The top three oil producing countries are Saudi Arabia, Russia and the United States. About 80% of the world's readily accessible reserves are located in the Middle East, with 62.5% coming from the Arab 5: Saudi Arabia (12.5%), UAE, Iraq, Qatar and Kuwait. However, with today's oil prices, Venezuela has larger reserves than Saudi Arabia due to crude reserves derived from bitumen (Soleimani *et al*., 2007). The presence of oil has significant social and environmental impacts, from accidents and routine activities such as seismic exploration, drilling and generation of polluting wastes not produced by other alternative energies.

Oil extraction is costly and sometimes environmentally damaging, over 70% of the reserves in the world are associated with visible macroseepages, and many oil fields are still found due to natural seeps. Offshore exploration and extraction of oil disturb the surrounding marine environment. Extraction may involve dredging, which stirs up the seabed, killing the sea plants that marine creatures need to survive. But at the same time, offshore oil platforms also form micro-habitats for marine creatures (Mohebali *et al*., 2008).

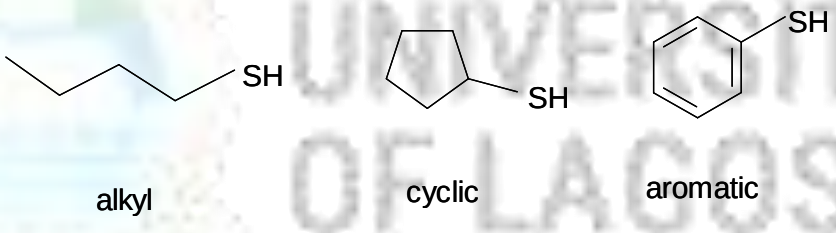
Burning oil releases Carbon (IV) oxide (CO_2) into the atmosphere, which has been argued to contribute to global warming. Per joule, oil produces 15% less CO_2 than coal, but 30% more than natural gas. However, the unique role of oil as the main source of transportation fuel makes reducing its CO_2 emissions a difficult problem. While large power plants can, in theory, eliminate their CO_2 emissions by techniques such as carbon sequestering or even use them to increase oil production through enhanced oil recovery techniques, these amelioration strategies do not generally work for individual vehicles (Kilbane and Le Borgne, 2004).

There are two main ways to measure the oil consumption rates of countries: by population or by gross domestic product (GDP). This metric is important in the global debate over oil consumption/energy consumption/climate change because it takes social and economic considerations into account when scoring countries on their oil consumption/energy consumption/climate change goals. Nations such as China and

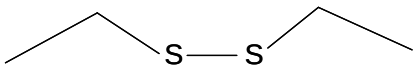
India with large populations tend to promote the use of population-based metrics, while nations with large economies such as the United States would tend to promote the GDP-based metric (Mohebali *et al*., 2007a).

2.4 SULFUR CONTENT OF PETROLEUM

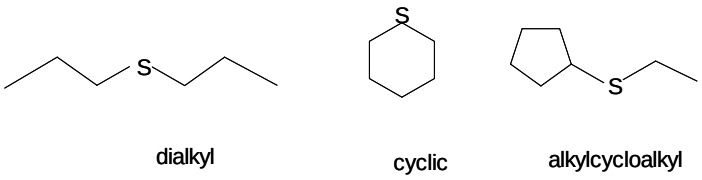
Crude oils are known to contain varying amounts of inorganic elements namely sulfur, nitrogen, oxygen and trace metals apart from the hydrocarbon components. Sulfur is present in organic forms as thiols, mercaptans, sulfides, benzothiophenes, polysufides, and also in inorganic forms like elemental sulfur, hydrogen sulfide (H_2S) and pyrites (Rall *et al*., 1972).



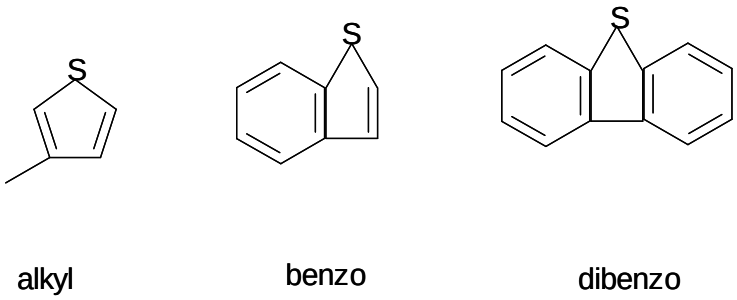
(a) Mercaptans (Thiols).



(b) Organic Disulfides



(c) Sulfides.



(d) Thiophenes

Figure 2.1 Chemical Structures of Organo-Sulfur Compounds in Crude Oil.

As a rule, the proportion, stability and complexity of sulfur compounds are greater in heavier crude oil fractions (Monticello, 2000). H_2S is a primary contributor to corrosion in refinery process units and combustion of sulfur-containing petroleum products can produce undesirable by-products such as sulfuric acid and sulfur dioxide. The sulfur content of crude oil can vary from 0.03 to 7.89 % (w/w) (Kilbane and Le Borgne, 2004). In general, as API gravity decreases, sulfur content increases. For example, light US crude (Rodessa, Louisiana) has an API gravity of 42.8 and a sulfur content of 0.28% w/w while extremely heavy crude from Venezuela has an API gravity of 9.5 and contains 5.25% w/w sulfur. The API gravity of oil is decreasing and sulfur content is increasing (Gray *et al.,* 2003), resulting in an increase in sulfur concentrations in finished petroleum products. Sulfur is preferentially associated with the higher molecular mass components of crude oils. The organosulfur compounds found in crude oil are generally classified into two types: non-heterocyclics and heterocyclics. The former comprise thiols, sulfides and disulfides. Cyclic or condensed multicyclic organosulfur compounds are referred to as sulfur heterocyclics. Thiophenic sulfur is present in many types of these compounds.

Table 2.2: Organic Sulfur Content in Crude Oils (De Krom, 2002)

Source	**Weight % Sulfur**
Argentina	0.06 – 0.42

Australia	0 – 0.1
Canada	0.12 – 4.29
Cuba	7.03
Denmark	0.2 – 0.25
Egypt	0.04 – 4.19
Indonesia	0.01 – 0.66
Iran	0.25 – 3.23
Iraq	2.26 – 3.3
Italy	1.98 – 6.36
Kuwait	0.01 – 3.48
Libya	0.01 – 1.79
Mexico	0.9 – 3.48
Nigeria	0.04 – 0.26
Norway	0.03 – 0.67
Russia	0.08 – 1.93
Saudi Arabia	0.04 – 2.92
United Kingdom	0.05 – 1.24
United States	0.29 – 1.95
Venezuela	0.44 – 4.99

An overview of the level of organic sulfur compounds present in crude oils found globally is given in Table 2.2

It is pertinent to observe that the Middle East and Venezelua have the most oil reserves with high sulfur contents (De Krom, 2002). More than 200 different sulfur compounds have been identified from crude oils. The distribution and amount of organic sulfur compounds reflect the source and maturity of the crude oils (Ho *et al*., 1974). Chemically immature oils are rich in sulfur and often have a high content in non-thiophenic sulfur compounds. During maturation labile non-thiophenic compounds are degraded and the sulfur content decreases (Payzant *et al*., 1986). Mature oils contain mainly high molecular weight alkylated benzo- and

dibenzothiophene derivates, the benzothiophene: dibenzothiophene ratio decreases with maturity (Tissot *et al*., 1984). The majority of the alkylated benzothiophenes can be found in the boiling range of 220 to 300^0C, then alkylated dibenzothiophenes are found in the boiling range of 350^0C (Schultz *et al*, 1999)

The most abundant form of sulfur in petroleum is usually thiophenic. Thiophenic sulfur often constitutes 50–95 % of the sulfur in crude oil and its fractions, and alkylated derivatives of dibenzothiophene (DBT) are the most common organosulfur compounds typically found in crude oil and fractions used to produce diesel (Kilbane and Le Borgne, 2004). DBT and its derivatives can account for a significant percentage of the total sulfur content of particular crude oils. In the middle distillate (diesel range) fractions, the sulfur that remains after conventional hydrodesulfurization treatment is typically in the form of C_x-DBT compounds.

When crude oil is refined, the sulfur concentrates into the high molecular mass fractions. A typical flue gas from the combustion of fossil fuels will contain quantities of NO_x, SO_x and particulate matter. SO_2 gas at elevated levels can cause bronchial irritation and trigger asthmatic attacks in susceptible individuals. Potential health risks expand to a broader section of the public when the gas turns to particulate matter. Long-term exposure to combustion-related fine particulate air pollution is an important environmental risk factor for cardiopulmonary and lung cancer mortality (Pope *et al.,*, 2002). Crude oils high in sulfur are designated “sour crude oils”, and those low in sulfur are “sweet crude oils”. Sulfur is removed during refining by catalytic hydro treating or by caustic wash (sweetening) processes.

Nitrogen exists as anilines, pyridines, quinolines, pyrols, carbazoles, benzonitrils and amides. Nitrogen is found in lighter fractions as basic compounds and in heavier fractions as non-basic compounds. Total nitrogen contents varies from <0.01% -1.0% by elemental analysis (Le Borgne and Quintero, 2003). Oxygen compounds are generally phenols, ketones and carboxylic acids. Metals found in crude oil include nickel, iron, vanadium, and arsenic in small quantities. These are removed during refining to avoid poisoning of catalysts, and when burning heavy fuel oils, to minimize

deposits of vanadium oxide, and nickel oxide in furnace boxes, ducts and tubes. Inorganic salts such as magnesium chloride or calcium chloride are suspended as minute crystals in crude oil or dissolved in entrained water (brine). These salts are removed or neutralized prior to processing to prevent catalyst poisoning, equipment corrosion and fouling (Kilbane, 2006).

2.5 LEGISLATIVE REGULATIONS ON SULFUR LEVEL IN CRUDE OIL FRACTIONS

The simplest way to decrease the amount of SO_2 emitted into the air is to limit the amount of sulfur in fuel. Legislative regulations in many countries call for the production and use of more environmentally friendly transportation fuels. Prior to 2003 the US EPA proposed that non-road diesel fuel sulfur be reduced from 3400 ppm to 500 ppm by 2007 (Song, 2003), and the European Union declared that the sulfur concentration in diesel fuel must reduce to <50 ppm by 2005 (Marcelis, 2002) and <10 ppm by 2009 (European Directive 2003/17/CE). The maximum allowable sulfur content for diesel in the USA is currently 15 ppm, with a target of 10 ppm by 2010 (Kilbane, 2006; Song, 2003). New and more effective approaches are needed for producing affordable ultra-clean (ultralow-sulfur) transportation and non-road fuels, because meeting the new government sulfur regulations in 2006–2010 represents only a milestone (Song, 2003).

2.6 HYDRODESULFURIZATION

Hydrodesulfurization (HDS) is a conventional chemical technique used specifically to remove sulfur from crude oils. It is an established physico-chemical method employed to reduce organically bound sulfur down to specified levels (Speight, 1981). The typical HDS operates at a temperature of 200–450°C and employs a pressure of 5 to 10 MPa in the presence of an inorganic catalyst (Orr, 1978; Speight, 1980), depending upon the level of desulfurization required (Speight, 1981). The

removal of the bulk of sulfur present in gas oil is conventionally performed by hydrotreatment with concurrent downward flow of hydrogen gas and hydrocarbon over a catalyst bed, known as the trickle bed concept. In the presence of a catalyst, hydrogen gas reacts with the sulfur compounds to produce gaseous hydrogen sulfide. In addition, this technique does not work well on certain sulfur molecules in oil, particularly the polyaromatic sulfur heterocycles found in heavier fractions. Dibenzothiophene (DBT) and DBTs bearing alkyl substitutions (Monticello and Finnerty 1985) that are considered as model compounds for the organic sulfur component of fossil fuels are difficult to remove using hydrodesulfurization. Moreover, this technique also results in the release of Carbon(IV)oxide. Thus, hydrodesulfurization is energetically costly and highly polluting (Gupta *et al.,*, 2005).

For the desulfurization of benzothiophene (BT) two different parallel reactions with hydrogen are catalyzed, known as the hydrogenation and the hydrogenolysis pathway. In the hydrogenation pathway shown in Figure 2.2, the thiophene ring is hydrogenated prior to desulfurization, while in hydrogenolysis pathway; the thiophene ring is split due to the attack of surface adsorbed hydrogen at the sulfur atom (Van Parijs *et al*., 1986). The desulfurization products are styrene and ethylbenzene. The H_2S formed inhibits the hydrogenolysis but not the hydrogenation reactions.

Houalla *et al*. (1980) proposed a mechanism for the HDS of DBT which states that the conversion proceeds via the path of minimal H_2 consumption, the hydrogenation of biphenyl and cyclohexylbenzene (CHB) proceeds slowly. The rate of DBT hydrogenation increased at higher H_2S concentration at the expense of hydrogenolysis.

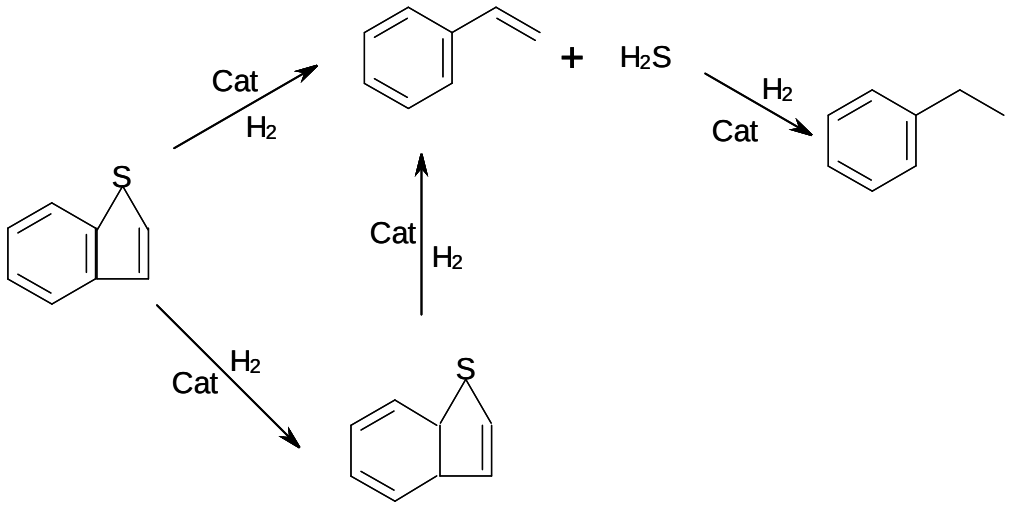


Fig. 2.2: HDS Reaction Mechanism for Benzothiophene (Cat = catalyst).

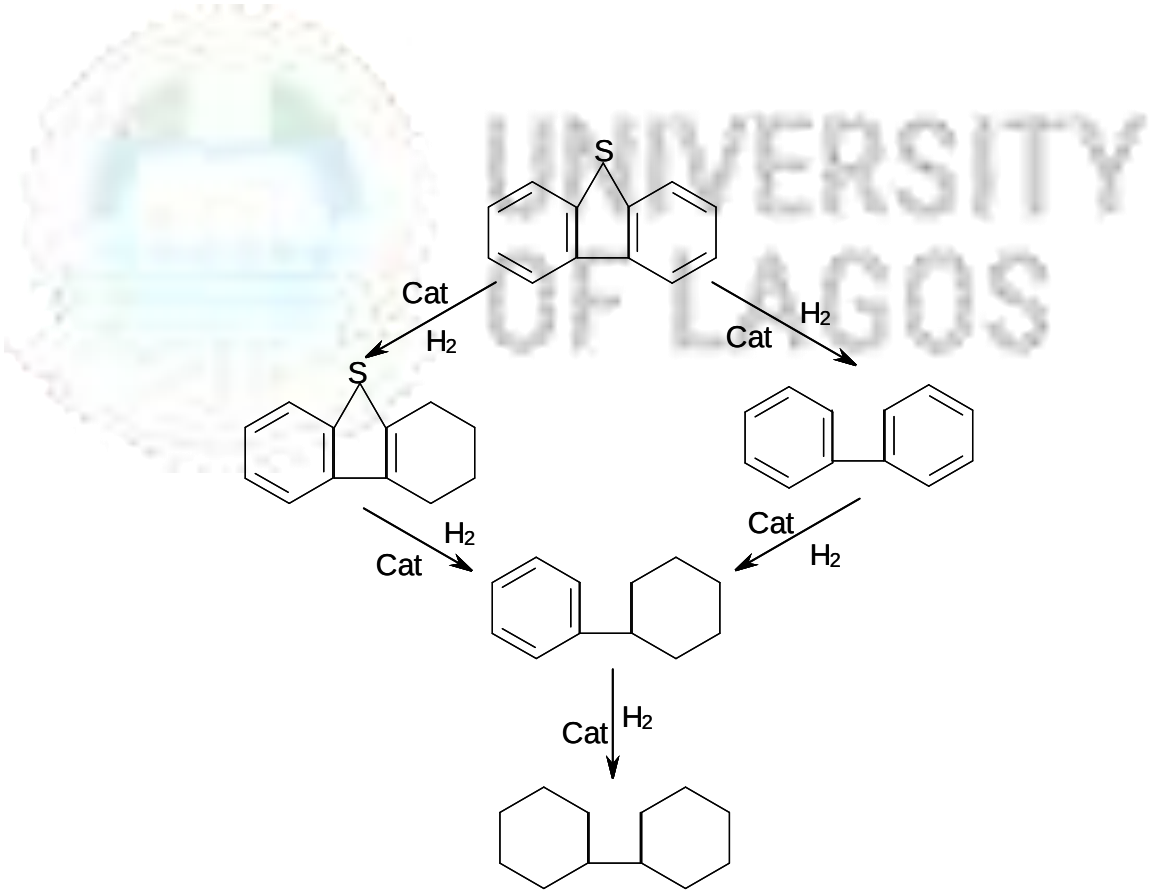


Figure.2.3: Proposed Reaction Mechanisms for DBT Hydrodesulfurization (Cat = catalyst).

Furthermore, the CHB concentrations depend on the applied catalyst type.

The presence of alkyl substituents on (di) benzothiophene molecules might favor one of the possible HDS routes; this will depend on the alkyl substituent position and thus to what extent the electron density is altered by the electron donating effect of alkyl groups (Schulz *et al*., 1999). In addition, substituents in the vicinity of the sulfur atom can cause steric hindrance and influence the HDS route (Kabe *et al*, 1992).

2.6.1 Catalysts

Dibenzothiophene (DBT) and alkyl substituents adjacent to the sulfur atom are refractory to HDS using conventional catalysts. The key sulfur compounds present after conventional HDS are include 4-methyldibenzothiophene (4-MDBT) and 4, 6-dimethyldibenzothiophene (4, 6-DMDBT). The γ-alumina (γ-Al_2O_3) supported molybdenum oxide catalyst promoted with cobalt or nickel have been widely used in conventional HDS processes (Segewa *et al*., 2000). Active sites are formed when MoO_3 changes to MoS_2 by sulfurization (Arnoldy *et al*., 1985). The question arises if these catalysts type are the most effective to use for deep desulfurization. The hydrogenation route is the most important pathway in the HDS of DBT with molecules with substituents at the 4- and 6- positions (Kabe *et al*., 1993). The direct hydrogenolysis is less favorable due to steric hindrance (Robinson *et al*., 1999a). The molecule becomes more flexible upon hydrogenation of one of the aromatic rings and the steric hindrance is relieved (Landau *et al*., 1996). Consequently, catalysts with a relatively high hydrogenation activity must be considered. Nickel promoted mixed sulfide catalysts are known for their high hydrogenation activity (Van Veen *et al*., 1993). Furthermore, noble catalysts (containing Pd or Pt) are attractive to use, because of their high hydrogenation activity (Robinson *et al*., 1999b). Kabe *et al*. (2001) reported that under deep desulfurization conditions, the partial pressure of H_2S has a strong inhibitory effect on the catalytic activity and product selectivity of HDS of DBT and 4,6-DMDBT. The inhibiting effect is the result of the more strongly adsorption of H_2S compared to DBT and 4, 6-DMDBT on the catalyst and thus dependent on the catalyst type. Noble catalysts are characterized by sensitivity for elevated H_2S level (Stanislaus *et al*., 1994). If deep desulfurization is performed in a different process stage, that is, after initial removal of the bulk of organic sulfur, alternative catalyst type can be applied, because high H_2S concentrations are

minimized. Robinson *et al*. (1999a) evaluated the role of the catalyst support in deep HDS. When NiMo was supported on amorphous silica-alumina (ASA) instead of alumina (γ-Al_2O_3), hydrogenation of 4-MDBT and 4E6M-DBT (4-ethyl, 6-methyl-dibenzothiophene) could be increased. Robinson *et al*., (1999b) has shown that ASA supported with NiW and Pt catalysts showed a much better performance of 4E6M-DBT desulfurization than ASA supported with NiMo, because of their high hydrogenation activity, especially at low H_2S level. When pre-hydrotreated diesel is subjected to deep hydrodesulfurization, other competing reactants are present which complicate the interpretation of single component model studies (Reinhoudt *et al*., 1999). In the study of Reinhoudt *et al*. (1999) it was shown that ASA supported with Pt/Pd catalysts are promising to apply in deep desulfurization, provided that H_2S is removed efficiently. A major drawback is the price of the noble metals.

During HDS, the catalysts will age and deactivate as a result of coke formation and metal deposition on the catalysts (Seki *et al*., 2001). The deposition severity is greatly influenced by the feedstock properties. As asphaltenes are precursors for coke formation higher boiling point fractions increase the deactivation. Next to that the HDS reaction conditions, temperature (regarding coke formation) and pressure (regarding metal deposition), enhance deactivation.

2.6.2 Development in HDS Reactor Configurations

Apart from the catalysts type involved, optimal process configurations to minimize the suppression of the H_2S on the catalyst activity are important. The H_2S produced from sulfur compounds with high reactivity in the early stage of desulfurization negatively influences HDS of less reactive sulfur compounds. To circumvent this problem, a two-stage principle carried out in conventional concurrent trickle-flow reactor can be applied. After removal of the bulk of easily convertible sulfur compounds in the first step, the more refractory compounds are removed in the second step with pure hydrogen (Ma *et al*., 1994, Reinhoudt *et al*., 1999). A more favorable H_2S profile during HDS can be achieved with countercurrent flow. However, the flooding produced at the required liquid velocity prevents the down flow of liquid against the

up flowing of gas (Sie, 1999). To operate below the flooding limit, the catalyst diameter must be increased leading to unacceptable pore diffusion limitations (Hanika *et al*., 1992). Reactors using monolithic catalyst supports may be an attractive alternative to conventional multi-phase reactors (Kapteijn *et al*., 2001). Instead of a catalyst trickle-bed, monolithic channels are present where bubble-train (or Taylor) flow occurs. Gas bubbles and liquid slugs move with constant velocity through monolithic channels approaching plug flow behavior. Gas is separated from the catalyst by a thin liquid film and during their travel through the channels the liquid slug shows internal recirculation. These two properties result in optimal mass transfer properties (Kapteijn *et al*., 2001). Apart from that, very sharp residence time distributions for gas and liquid compared to trickle flow can be achieved (Nijhuis *et al*., 2001). Currently, the application of monoliths in various forms and application is an object of research (Kapteinj *et al*., 2001). Larger channel geometries (internally finned monolith channels) might allow countercurrent flow at a relevant industrial scale and the scale up properties are promising.

In summary, hydrodesulfurization of petroleum feedstock for a low-sulfur product requires a larger reactor volume, longer processing times, and substantial hydrogen and energy inputs. Deep HDS technology results in various problems in the process, which limit its usefulness:

(i) the application of extreme conditions to desulfurize refractory compounds results in the deposition of carbonaceous coke on the catalysts;
(ii) exposure of crude oil fractions to severe conditions including temperatures above about 360 °C decreases the fuel value of treated product;
(iii) deep HDS processes need large new capital investments and/or have higher operating costs;
(iv) the H_2S that is generated poisons the catalysts and shortens their useful life;
(v) deep HDS is affected by components in the reaction mixture such as organic heterocompounds and polyaromatic hydrocarbons (Egorova, 2003).

Moreover, this technique also results in the release of Carbon(IV)oxide. Thus, HDS is energetically costly and causes global warming with its attendant problems. The high cost and inherent chemical limitations associated with HDS make alternatives to this technology of interest to the petroleum industry. Moreover, current trends toward stricter regulations on the content of sulfur in fuels provide incentive for the continued search for improved desulfurization processes (Folsom *et al.,*, 1999). Organic sulfur in petroleum fossil fuels is present in a myriad of compounds, some of which are unstable in that they cannot readily be desulfurized or are refractory because they do not easily yield to conventional desulfurization treatment by HDS. Increasing the severity of HDS also elicits undesirable effects on fuel quality as other chemical components are reduced at the higher temperatures and pressures needed to achieve low sulfur levels (Konishi *et al.,*, 2000).

2.7 BIODESULFURIZATION

In middle distillate (diesel range) fractions, the sulfur that remains after aggressive HDS treatment is typically in the form of Dibenzothiophene (DBT) and its substituent compounds. The most refractory DBTs have substituents at the 4 and 6 positions, which are adjacent to the sulfur moiety and are believed to sterically hinder access of the sulfur atom to the catalyst surface (Kabe *et al.,*, 1992). As regulations on sulfur levels in fuels become stricter, more of the HDS-refractory compounds must be removed. As a result, HDS-refractory sulfur compounds represent a significant barrier to reaching very low sulfur levels in the middle and heavy distillate range fuels.

To overcome these disadvantages, a biotechnological approach was considered as an alternative. Biotechnology refers to the use of living organisms or a part of an organism to modify or make products, improve plants and animals, or develop microorganisms for specific uses. Biological catalysts operate in a wide range of conditions, including ambient temperature and pressure, and are endowed with high selectivity, resulting in decreased energy costs, low emission, and no generation of undesirable side-products. Whole microorganisms as well as their enzymes can use

a wide range of compounds as substrates transforming them into specific products. Because of these unique characteristics, biotechnology is thought to be an interesting alternative or complement for the development of refining technology for fossil fuels. Microorganisms are reported to have the abilities to remove sulfur from fossil fuels in a process called biodesulfurization. The use of microorganisms might offer an alternative way to remove sulfur specifically from hydrocarbon fractions without altering the carbon skeleton. Biodesulfurization is considered an environmentally benign process because of the mild process conditions (low pressure and temperature). However, conversion rates are expected to be lower than that of the aforementioned HDS technique.

Therefore, biodesulfurization can be considered a complimentary process, after the bulk sulfur has been removed using HDS techniques. The cleavage of the Carbon - Sulfur bonds can be performed either with an aerobic or an anaerobic mechanism. Based on the bond strengths of the C – S bonds in the sulfur heterocycles (thiophene, benzo- and dibenzothiophene) will be broken preferentially (Bresslar *et al*., 1998).

The C – C bond strengths are greater compared to the C – S bonds. As can be seen from Table 2.3, the addition of oxygen to a carbon atom adjacent to a sulfur atom weakens the bond strengths thus making the C – S bond more susceptible to cleavage. This is a common feature in the aerobic conversion of sulfur compounds, where enzymes (dioxygenases) introduce oxygen to facilitate the C – S cleavage.

According to the bond strength in Table 2.3, the C – S bonds will also be attacked preferentially in the anaerobic reaction mechanism that is similar to the metal-catalyzed HDS reaction mechanism. (Marcelis, 2002).

Biodesulfurization can be broadly classified into three different categories according to their mode of action: oxidative C–C cleavage, oxidative C–S cleavage and reductive C–S cleavage. These categories are discussed below.

Table 2.3: Bond Strengths of various C – S, C – C and C – H Bonds
(Adapted from Bresslar *et al*., 1998)

C – S Bonds	**Strength (KJ/Mol)**
C – S in Thiophene	341
C – S in Benzothiophene	339
C – S in Dibenzothiophene	338
HS – CH_3	312
H_3C – SCH_3	308
H_3C – SO_2CH_3	280
H_3C – $SCH_2C_6H_5$	257
H_3C – $SO_2CH_2C_6H_5$	221
C – C Bonds	**Strength (KJ/Mol)**
H_3C – CH_3	376
H_2C = CH_2	733
C – C in Benzene	505
H_3C – CH_2CH_3	330
H_3C – $COCH_3$	290
C – H Bonds	**Strength (KJ/Mol)**
H – CH_3	438
H – CH_2OH	410
H – CHO	364

2.7.1 Oxidative C–C cleavage

Early work on biodesulfurization focused on organisms that degrade DBT. Kodama *et al*. (1970 and 1973) first reported the oxidative cleavage of DBT. They identified various intermediates which are generated during the oxidation and proposed the

well known Kodama pathway. It involves three major steps: hydroxylation, ring cleavage and hydrolysis.

Various microorganisms are known to follow this pathway. The two microorganisms reported in 1970 were *Pseudomonas jijani* and *P. abikonesis* (Kodama *et al*., 1970), which form the basis of the Kodama pathway (Figure 2.4). Degradation of DBT is plasmid encoded in these microorganisms and is encoded on a 55- MDa plasmid (Monticello *et al*., 1985). The product of DBT oxidation is inhibitory to both cell growth and further DBT oxidation. It is reported that DBT oxidation is induced by naphthalene or salicylic acid (Kodama, 1977; Monticello *et al*., 1985) and to a much lesser extent by DBT and is repressed by succinate. In addition to the Kodama pathway, *P. putida* follows an alternate pathway as well (Mormile and Atlas 1989), in which DBT is degraded to DBT sulfone. The organism first metabolizes DBT via the Kodama pathway and then transforms DBT via the alternate DBT sulfone pathway. Similar observations were also made in the case of the fungi, *Cunninghamella elegans* (Crawford and Gupta, 1990) and *Pleurotus ostreatus* (Bezalel *et al*. 1996) that oxidized DBT to DBT sulfoxide and DBT sulfone as dead end products.

The same pathway is also reported to be followed by *Rhizobium meliloti* (Frassinetti *et al*. 1998) and *Beijerinckia* sp. (Laborde and Gibson, 1977).

In the case of *Beijerinckia sp*., pyruvate is also formed, along with 3-hydroxy-2-formyl benzothiophene (HFBT). Although HFBT is reported as the end-product of DBT degradation via the Kodama pathway, there are instances where other possible products of HFBT have been reported. Eaton and Nitterauer (1994) showed that benzothiophene-2, 3-dione was formed from 2-mercaoptophenylglyoxylate by acid-catalyzed dehydration due to the prevalence of acidic conditions during the extraction of culture supernatants. It has also been reported that HFBT can be degraded to Carbon(IV)oxide (Bressler and Fedorak, 2001). Substituted DBTs are more common in hydrodesulfurization-treated diesel. In view of this, aerobic metabolism of methyl DBTs were also studied in *Pseudomonas* strains (Saftić *et al*. 1993). These strains metabolized DBT to benzothiophene- 2, 3-dione, HFBT, DBT sulfoxide and sulfone.

Two pathways of DBT degradation in *P. putida* was observed when grown on methyl DBTs, the unsubstituted ring was oxidized and cleaved by the Kodama pathway to form the corresponding methyl HFBT and benzothiophene-2,3- diones in addition to methyl DBT sulfones and DBT methanols. Transformation of dimethyl DBTs was studied using pure cultures of *Pseudomonas* strains BT1, W1, and F, and four petroleum-degrading mixed cultures (Kropp *et al*. 1997). The unsubstituted rings were degraded in 3, 4-dimethyl DBT to give 6, 7-dimethyl HFBT and 6, 7-dimethylDBT-2, 3-dione among other products. The 4, 6- dimethyl DBT was not oxidized in substantial amounts by any of the cultures.

This suggested that the Kodama pathway enzymes preferred to cleave the unsubstituted homocyclic rings, as compared with their methylated derivates. Studies on the degradation of HFBT, DBT sulfoxide and DBT sulfone by mixed culture (Mormile and Atlas 1988) showed a depletion of HFBT. In the case of DBT sulfoxide and DBT sulfone no sulfate was released into the medium but there was production of Carbon(IV)oxide thus indicating the degradation of these compounds but not complete demineralization.

2.7.1 Molecular Biology of the Kodama Pathway

Denome *et al*. (1993) showed that a single genetic pathway controls the metabolism of DBT to HFBT and the metabolism of naphthalene to salicylaldehyde. A 9.8- kb DNA fragment was cloned and sequenced from *Pseudomonas* strain C18 that encoded DBT biodegradation genes. The sequence contained nine open reading frames (ORFs), designated dox ABDEF GHIJ. The naphthalene dioxygenase-encoding *P. putida* genes ndoA–ndoC was identical to three of the ORFs (doxA, doxB, doxD). These bacteria can also use a range of other aromatic hydrocarbons, like methyl DBTs (Saftić *et al*. 1993; Kropp *et al*. 1997) and naphthalene. Thus, it appears that there may not be a separate set of genes or enzymes for DBT degradation via the Kodama pathway. The similarity of the dox genes with the ndo genes (Denome *et al*. 1993) and the ability of the naphthalene dioxygenase from the *Pseudomonas* strain NCIB 9816-4 to oxidize DBT further suggest that DBT (Resnick

and Gibson, 1996) may simply serve as an alternate substrate for naphthalene degrading enzymes.

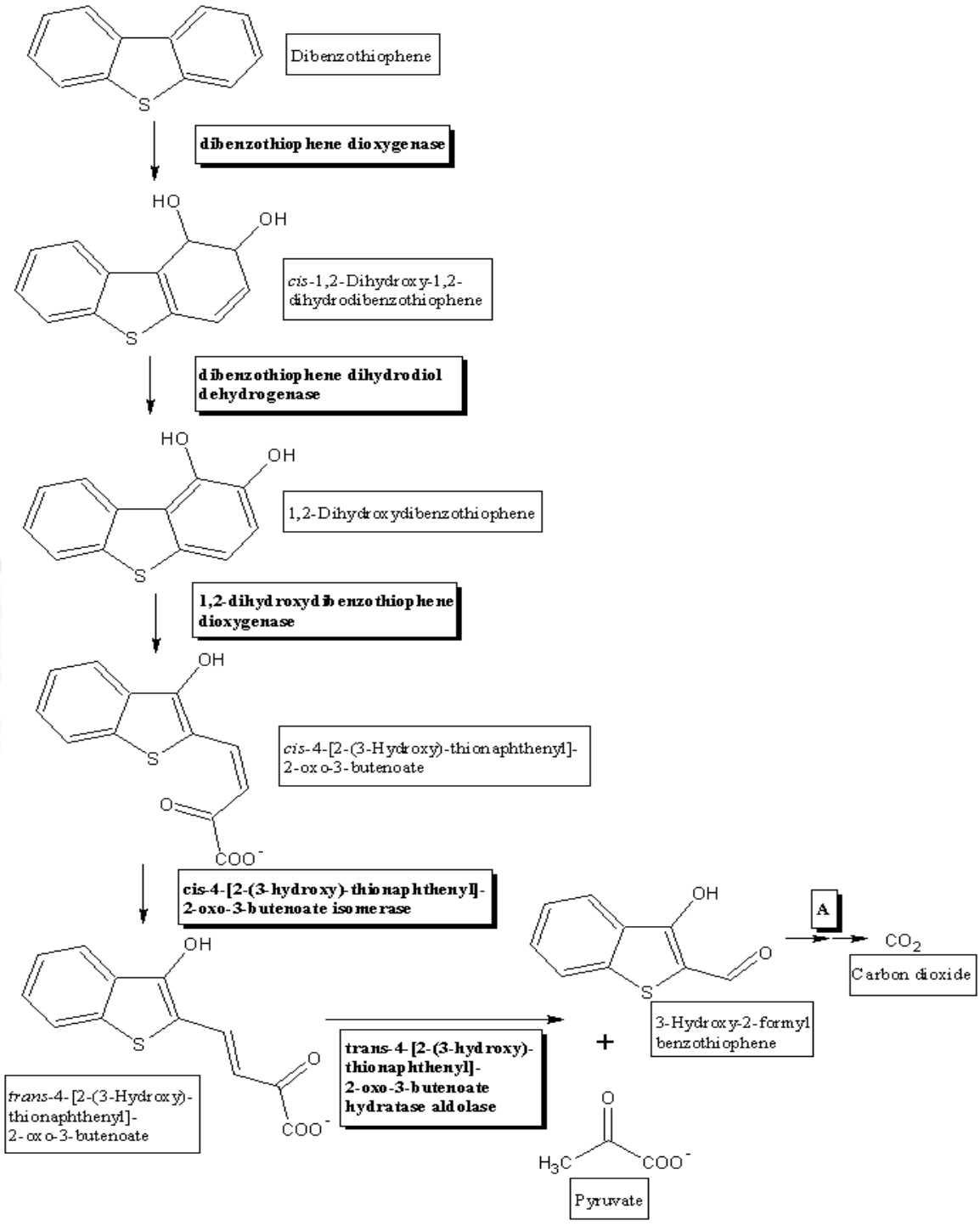


Figure 2.4: Proposed Pathway of Dibenzothiophene Metabolism by *Pseudomonas abikonesis & Pseudomonas jijani* (The Kodama pathway)

2.7.2 Oxidative C–S Cleavage

This process removes sulfur from DBT and methyl DBT in a sulfur-specific manner without affecting the carbon skeleton thus preserving the fuel value (Figure 2.8) Thus, because its specificity for sulfur atoms and of operation under aerobic conditions, studies on biodesulfurization are focused on this technique for the past 10 years. This process for sulfur removal was first reported for *Rhodococcus rhodochrous* IGTS8 in 1993 by Gallagher *et al*. (1993). During this process of conversion of DBT to 2-hydroxybiphenyl, four different molecules are formed. So, this pathway is known as the 4S pathway. Besides *R. rhodochrous* IGTS8 (Kilbane and Jackowski, 1992), other bacteria also reported to follow this 4S pathway are *R. erythropolis* D1 (Izumi *et al*., 1994; Ohshiro *et al*., 1994), *Rhodococcus* ECRD1 (Grossman *et al*., 1999), *Rhodococcus* B1 (Denis-Larose *et al*., 1997), *Rhodococcus* SY1 (Omori *et al,*. 1992), *Rhodococcus* UM3 (Purdy *et al*., 1993), *Gordona* CYKS1 (Rhee *et al*. 1998), *Xanthomonas* (Constanti *et al*., 1994), *Nocardia globelula* (Wang and Kraweic ,1994), *Paenibacillus* strain (Konishi *et al*., 1997), and *Mycobacterium* sp. (Li *et al*., 2003). The 4S pathway proceeds via two cytoplasmic monooxygenases (DszC, DszA) supported by flavin reductase (DszD) and a desulfinase (DszB). DBT monooxygenase (DszC) catalyzes the sequential conversion of DBT to DBT sulfoxide and DBT sulfone, this is shown schematically in Figure 2.5.

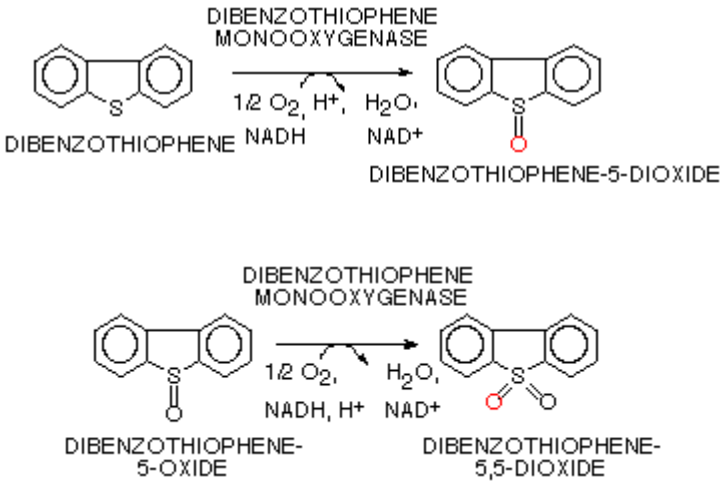


Figure 2.5: Equation of the Reaction for the Conversion of DBT to Sulfone

Figure 2.6 shows how DBT-5, 5-dioxide 4 monooxygenase (DszA) catalyzes the transformation of DBT sulfone to DBT sulfinate, also utilizing $FMNH_2$ as a co substrate with a reaction rate 5- to 10-fold higher than DszC.

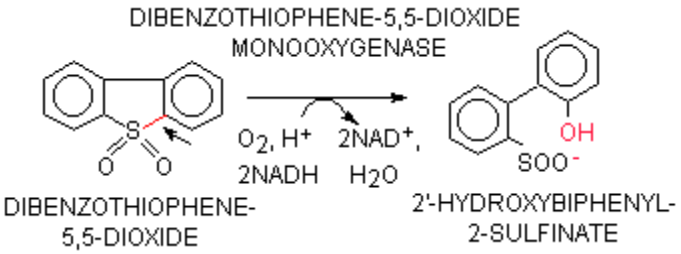


Figure 2.6: Equation of Reaction Showing the Cleavage of the First C— S Bond

DszB, an aromatic sulfinic acid hydrolase, affects a nucleophilic attack of a base-activated water molecule on the sulfinate sulfur to form 2-hydroxybiphenyl (2-HBP). Figure 2.7 depicts this reaction.

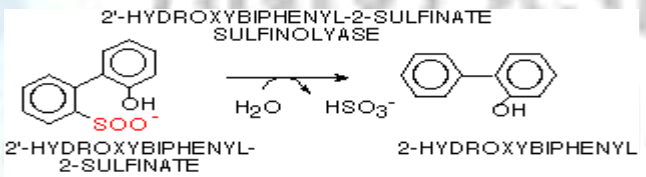


Figure 2.7: Equation of Reaction Showing the Liberation of Inorganic Sulfur

In nature, the cell has achieved its goal. It has the sulfur it needs to grow. The sulfite can be reduced to sulfide and incorporated into sulfur-containing amino acids and vitamins necessary for growth.

The oxygen atom incorporated at each step of this desulfurization pathway is derived from molecular oxygen. DszC and DszA genes do not use NADH directly (Gray *et al*., 1998) but use $FMNH_2$ from a FMN: NADPH oxidoreductase (DszD). DszD couples the oxidation of NADH with substrate oxygenation by DszA and DszC. Most of the strains reported are mesophiles, i.e., the DBT-desulfurizing ability is high only around 30°C and decreases with higher temperatures (Figure 2.5). Hydrodesulfurization-

treated diesel oil is at a temperature much higher than 30°C and a cooling system is necessary for the practical use of these DBT desulfurizing bacteria. If biodesulfurization could be performed around 50°C, it would be unnecessary to cool the hydrodesulfurization-treated diesel oil to ambient temperature. In addition, contamination by undesirable bacteria, which affects the biodesulfurization process, would be avoided at high temperature. Until now, only a few microorganisms, such as a *Paenibacillus* strain (Konishi *et al*., 1997), *Bacillus subtilis* WU-S2B (Kirimura *et al*., 2001), *Mycobacterium* sp. X7B (Li *et al*., 2003), *Mycobacterium* sp. GTIS 10 (Kayser *et al*., 2002), and *M. pheli* WU-F1 (Furuya *et al*., 2001) are reported to show desulfurization at high temperature. These thermophilic strains, in addition to being desulfurizing at the higher temperature range, have additional advantages compared with mesophiles. Thus, the *Paenibacillus* strain shows highest activity at 60°C and can also desulfurize substituted DBTs (Konishi *et al*., 1997, 2000b). *B. subtilis* can desulfurize both 2, 8-dimethyl DBT and 4, 6-dimethyl DBT and the reaction rate is same for both compounds (Kirimura *et al*. 2001). *Mycobacterium* GTIS 10 shows a higher tolerance towards 2-HBP and a desulfurizing ability comparable with that of *R. rhodochrous* IGTS8 (Kayser *et al*. 2002). *M. pheli* WU-F1 degrades asymmetric structural isomers of DBT, such as napthothiophene and 2- ethyl napthiophene in a sulfur-specific mode (Furuya *et al*. 2001). *Mycobacterium* X7B degrades 2-HBP further to 2-methoxybiphenyl, partially eliminating the inhibitory effect of the product and pollution from diesel oil.

2.7.3 Molecular Biology of 4S Pathway

The genes involved in DBT metabolism have been called biodesulfurization (Ohshiro *et al.,* 2005), Dsz (Piddington *et al.,* 1995), tds (Ishii *et al.,* 2000), mds (Nomura *et al.,* 2005) and sox (Denome *et al.,* 1994). Because several other unrelated genes have already been labelled *sox* and to avoid confusion with these other genes, the *sox* designation has been generally rejected. Biodesulfurization, Dsz, Tds and Mds have all been accepted as gene products. *R. erythropolis* IGTS8 (Gallardo *et al.,* 1997; Gray *et al.,*1996; Oldfield *et al.,* 1997; Piddington *et al.,* 1995) and *Rhodococcus* sp. X309 (Denis-Larose *et al.,* 1997) were among the first strains to be characterized at

the molecular level. The desulfurization (Dsz) phenotype was conferred by a 4 kb gene locus on the large plasmid (Denis-Larose *et al.,* 1997; Gray *et al.,* 1998; Oldfield *et al.,* 1997; Rambosek *et al.,*1999). The desulfurization ability of *Rhodococcus* sp. ECRD-1 appeared to be an exclusive property of a 4 kb gene locus on a large plasmid (Prince and Grossman, 2003). Although expressed as an operon, DszB is present at concentrations several-fold less in the cytoplasm, as compared with DszA and DszC (Li *et al*., 1996). These genes, when cloned on a Dsz− phenotype, confer the ability to desulfurize DBT to 2-HBP. The Dsz operon was found on a large plasmid of 150 kb in *R. rhodochrous* IGTS8 and on a 100 kb plasmid in other strains.

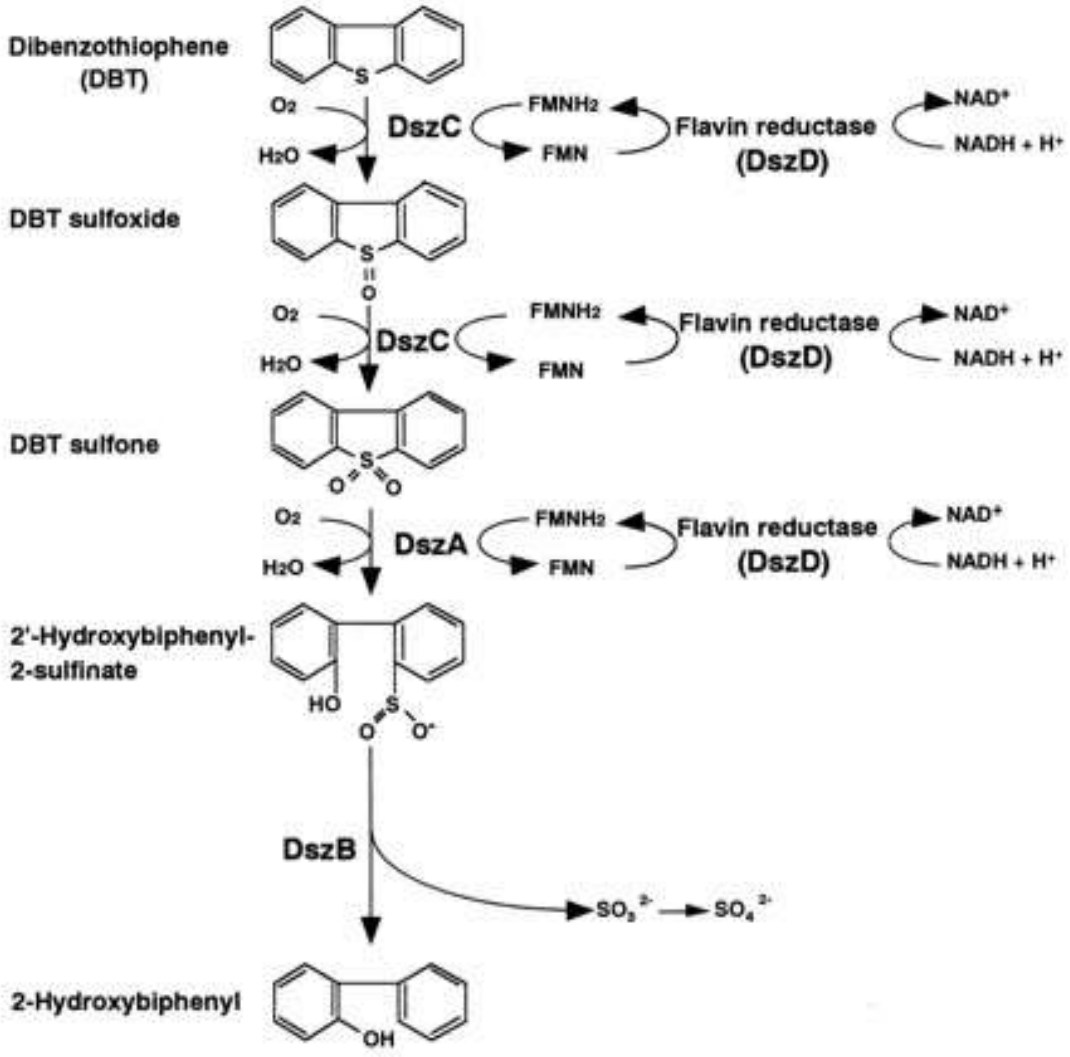


Figure 2.8: The Sulfur Specific Oxidative Pathway of DBT Desulfurization by Bacteria.

Figure 2.8 is the graphical presentation of combined reactions shown in Figures 2.5 to 2.7

Promoter and regulatory regions of the Dsz operon were also studied and it was found that enzymes are strongly repressed in the presence of readily bioavailable sulfur (Li *et al*., 1996), i.e., sulfate, sulfide, methionine and cysteine, a phenomenon that is similar to responses to other sulfur compounds under sulfur starvation. In *Rhodococcus* the *Dsz* genes are located near insertion sequences (Denis-Larose *et al.,* 1997; Kilbane and Le Borgne, 2004). Therefore, the desulfurization system is organized as one operon with three genes (DszA, DszB, and DszC) transcribed in the same direction and under the control of a single promoter (Gray *et al.*, 1998; Oldfield *et al.*, 1997). Matsubara *et al.* (2001) purified and characterized the flavin reductase from *R. erythropolis* D-1. The N-terminal amino acid sequence of the purified flavin reductase was identical to that of DszD of *R. erythropolis* IGTS8.

Accumulation of 2-HBP also inhibits growth and desulfurization. Monticello (1998) postulated that repression of the enzyme synthesis occurs at the transcription level, but 2-HBP does not act as an inhibitor of the enzyme. Analogous to the Dsz operon in mesophiles, the tds (thermal desulfurization (Ishii *et al*., 2000; Konishi *et al*., 2000b) operon is located on an 8.7-kb DNA fragment in the thermophile *Paenibacillus* sp. A11-2. The tds A, tds B, and tds C nucleotide sequences and the deduced amino acid sequence showed significant homology to the DszA, DszB and DszC genes of *R. rhodochrous* IGTS8. However, several local differences were observed between them. Several gram-positive and gram-negative organisms are known to have desulfurization genes; and they show 70% homology (McFarland, 1999). Efforts to increase the rate of sulfur removal from aromatic sulfur heterocycles have been possible due to the use of genetic engineering techniques or the use of immobilization matrices. Recombinant *Pseudomonas* strains have been designed to enhance their desulfurization activity by inserting the Dsz gene cluster in their chromosome (Gallardo *et al*., 1997). The recombinant strains have better desulfurization activity than the wild-type strain due to the production of surfactant by the host organism. It has been shown (Rambosek *et al*. ,1999) that the reconstruction

of several promoters containing the *R. rhodochrous* IGTS8 Dsz gene, with the DszD gene, helps increase the rate of DBT desulfurization. Rambosek *et al*. (1999) generated several promoter constructs that alleviated sulfur repression and increased desulfurization rate via the 4S pathway. The enzyme expression can also be considerably increased by the use of transposons for generating high-activity desulfurizing enzyme (Tanaka *et al*., 2002). For example, a mutant of *R. erythropolis* KA2-5-1 was constructed using a transposon that showed desulfurization activity even in the presence of sulfate. Sulfate repression can also be overcome by deleting the last gene of the metabolic pathway (DszB). Deletion of this gene eliminates the slowest step of the Dsz pathway and allows the accumulation of hydroxybiphenyl sulfinate, which is a more valuable product than the sulfate because it can be recovered from the aqueous phase and used as surfactant (Pacheco *et al*., 1999). The desulfurization rate can also be increased using directed-evolution methods including gene shuffling. A first approach consists in generating libraries of evolved dszC genes by a new in vitro DNA recombination method called “random chimeragenesis on transient templates” RACHITT (Coco *et al*., 2001; Pelletier, 2001). In this method, randomly cleaved parental DNA fragments are annealed to a transient polynucleotide scaffold. This technique appears to have advantages in the diversity it can generate, the fact that closely linked alleles are easily recombined and parental sequences are virtually assured to be absent in the recombinant pool. Simultaneous improvements of desulfurization rate and range of oxidized substrates were obtained by this method. Coco *et al*. (2001) recombined dszC genes derived from two different genera, *Rhodococcus* and *Nocardia*. The DBT-MO enzyme from *Nocardia* had a higher substrate affinity and that from *Rhodococcus* had higher specific reaction rate. Variants were isolated that had both a higher rate and a more extensive substrate oxidation. This study focused on DszC because this enzyme catalyzes the first step in desulfurization. Sterically hindered DBT derivatives rather than DBT are the main target molecule when extremely low sulfur levels are desired. Strains able to desulfurize substituted DBTs have been reported (Lee *et al*., 1995; Darzins and Mrachko, 2000). As for chemical catalysis, DBT substituted in positions 4 and 6 are the most difficult to desulfurize due to steric hindrance problems (Oshiro

et al., 1996). A genetically modified derivative of the *R. rhodochrous* IGTS8 strain, containing multiple copies of the Dsz gene was reported to desulfurize C5-DBT derivatives ten-times slower than DBT.

A finding in 2002 is that bacterial cultures that possess identical *Dsz* gene sequences can have very different Dsz phenotypes. This was clearly illustrated by examining the desulfurization activity of *Mycobacterium phlei* GTIS10 having *DszABC* gene sequences identical to *R. erythropolis* IGTS8; the temperature at which maximum desulfurization activity was detected in the cultures was about 50 °C and 30 °C, respectively (Kayser *et al.,* 2002; Kilbane, 2006). Characterization of four bacterial cultures capable of utilizing DBT as the sole source of sulfur revealed that these cultures had identical *Dsz* genes, but the cultures differed significantly with regard to their substrate range, desulfurization activity and yield of metabolites (Abbad-Andaloussi *et al.,* 2003). A comparative study of *M. phlei* SM120-1 and *M. phlei* GTIS10 aimed at broadening the understanding of the Dsz trait at intra-species level revealed considerable differences in the phenotypic and genotypic characteristics of these two desulfurizing strains (Srinivasaraghavan *et al.,* 2006). The range of Dsz phenotypes observed in different cultures may reflect the ability of each bacterial species or strain to provide cofactors and reaction substrates under the conditions tested. The transport of substrates and products might also contribute to desulfurization activity, as demonstrated by the fact that cell-free lysates of desulfurization cultures can exhibit a broader substrate range than the intact cell culture (Kilbane, 2006).

The 4S pathway is a complex enzyme system and its cofactor requirements prohibit the use of purified enzyme systems rather than whole cells for a practical biodesulfurization process (Kilbane and Le Borgne, 2004). Moreover, cell-free extracts exhibit a lower desulfurizing activity (Gupta *et al.,* 2005). Implementation of the biodesulfurization process consists of several stages, including (i) growing the selected strain in a suitable medium so as to obtain cells which exhibit the highest possible desulfurizing activity and (ii) harvesting these active cells and using them in the form of resting cells (biocatalysts) in a biodesulfurization process. The formulation

of the growth medium is important for production of a high density of resting cells, which express the highest level of desulfurization activity. In this context, sulfate contamination of the growth medium is the main barrier to the expression because the Dsz phenotype is repressed by sulfate (Ma *et al.,* 2006a).

To obtain large-scale production of desulfurizing resting cells, repression can also be avoided by various microbiological procedures, including lowering the repressor (sulfate) content of growth medium, substitution of sulfate by an alternative sulfur source, and production of resting cell biomass on sulfate or another suitable sulfur source followed by induction of the Dsz phenotype in the resting cells using DBT (Chang *et al.,*, 2001; Mohebali *et al.,* 2008). Because *Dsz* genes are repressed by inorganic sulfate, most researchers have used synthetic media containing DBT as the sulfur compound for growth. Desulfurizing activity has been shown to be enhanced by restricting the amount of DBT added to the medium (Yoshikawa *et al.,* 2002). Mass production of biocatalyst using DBT has been considered to be commercially impractical as a result of its high price and low water solubility, and growth inhibition by 2-HBP. Researchers have therefore tried to find a suitable alternative to DBT as a sulfur source for growing cells, for example 2-aminoethanesulfonic acid (Yoshikawa *et al.,*, 2002) and dimethyl sulfoxide (Mohebali *et al.,*, 2008).

2.7.4 Enzymology of the 4S Pathway

A lot of works on genetic studies have been done on the Dsz system but only a few reports are available on the purification and characterization of these enzymes. Dsz enzymes are known to be purified from *R. rhodochrous* IGTS8 (Gray *et al*. 1996), *R. erythropolis* D-1 (Takashi *et al*., 1997) and *Paenibacillus sp*. strain A11-2 (Konishi *et al*. 2000c). The DszA enzyme is widely studied and has been purified from all three strains (viz., *R. rhodochrous* IGTS8, *R. erythropolis* D-1, *Paenibacillus sp*. strain A11-2). It is a monooxygenase that oxidizes DBT sulfone to 2-(2′-hydroxyphenyl) benzene sulfinate (HPBS). Being a thermophile, the enzyme isolated from Paenibacillus sp. strain A11-2 (Konishi *et al*., 2000c) exhibits different characteristics from the enzyme isolated from other mesophiles. It is a homodimer with a subunit molecular weight of 50 kDa and the optimum pH and temperature for its function are pH 7.5 and 35°C,

respectively. It does not show any enzyme activity beyond 50°C but the enzyme isolated from the thermophile *Paenibacillus* sp. strain A11-2 has an optimum temperature of 45°C and is stable till 60°C. It works at an optimum pH of 5.5. The enzyme from Paenibacillus sp. strain A11-2 shows a higher activity towards bulkier substrates than its mesophilic counterparts, indicating the applicability of biodesulfurization to the processing of actual petroleum fractions but it loses its activity in the presence of thiol reagents showing the presence of thiol moiety at its active site. Monooxygenase enzymes in general show a 1.7-fold higher activity towards 4, 6-dimethyl DBT sulfone as compared to DBT sulfone. The activity of the enzyme from *R. erythropolis* D-1 (Takashi *et al*., 1997) is inhibited by 50% in the presence of 1 mM EDTA or any other chelating agent but no inhibition is observed in the case of *R. rhodochrous* IGTS8 (Gray *et al*., 1996), even in the presence of 10 mM EDTA. The DszB enzyme or desulfinase is the rate-limiting enzyme and catalyzes the conversion of HPBS to HBP. It is the least studied enzyme as very little amount is produced. It is a monomer with a subunit molecular weight of 40 kDa and shows enzyme activity over a wide temperature range (25–50°C), the optimum being 35°C (Watkins *et al*. 2003). The working pH range for this enzyme is 6.0–7.5. A cysteine residue is shown to be present in the sequence of this enzyme and it is found to be critical for enzyme activity. The third enzyme DszC or DBT monooxygenase catalyzes the conversion of DBT to DBT sulfone in a two step process with DBT sulfoxide being the intermediate compound. The first oxidation step (rate constant 0.06 min−1) is one-tenth of the rate of the second step (rate constant 0.5 min−1). This enzyme shows homology to the acyl coenzyme A enzyme and is a homotetramer with a subunit molecular weight of 50 kDa as reported by Gray *et al*. (1996). Ohshiro *et al*. (1994) isolated DszC from *R. erythropolis* D-1 and reported DszC to be a homohexamer with a subunit molecular weight of 45 kDa. Its activity is maximum at a temperature of 40°C and an optimum pH of 8.0. This enzyme can act on the derivatives of DBT such as 4,6-dimethyl DBT, 2,8-dimethyl DBT, 3,4-benzo-DBT but it does not show any activity on carbazole, dibenzofuran and fluorene i.e., DBT atoms substituted for sulfur atoms. Isotopic labeling studies indicated that the two oxygen atoms were derived from molecular oxygen. The DszD enzymes or

reductases are associated with monooxygenases since monooxygenases cannot work in the absence of these reductases. DszD was purified from the thermophilic strain *Paenibacillus* sp. strain A11-2 (Konishi *et al*., 2000c) and characterized to be a homodimer with a subunit molecular weight of 25 kDa. It shows similarity to a kinesin-like protein and has 100% homology to the Thc E protein, a group III-type alcohol dehydrogenase (oxidoreductase) named N-N′-dimethyl-3- nitrosoaniline oxidoreductase. This enzyme works well at 55°C and pH 5.5. All the monooxygenases showed a requirement for equimolar quantities of flavin reductase for their respective oxygenation reactions. Xi *et al*. (1997) studied the enhanced desulfurization activity of DszC and DszA under in vitro conditions with increased concentrations of flavin reductase, suggesting the two to be terminal oxygenases. The reaction rate with 1 unit ml^{-1} of flavin reductase was linear for 10–15 min whereas it was more than 20 min with a lower concentration of the same. In addition, the flavin was not found to be a cofactor for the monooxygenases, although the reduced form of the flavin acted as a substrate.

The desulfurization activity of naturally occurring bacterial cultures is low in comparison to the requirements of a commercial process. It has been estimated that a successful commercial process would require a biocatalyst with desulfurization activity of 1.2–3 mM DBT (g dry cell weight)$^{-1}$ h^{-1} as measured with petroleum products. To achieve this, the currently available biocatalysts require an increase in desulfurization rate of about 500-fold (Kilbane, 2006). The key to increasing the bacterial desulfurization rate is to identify the genes responsible for C–S bond cleavage in organic sulfur compounds and manipulate the system through genetic engineering techniques. Therefore, future biodesulfurization studies will focus on development of this promising research area. To develop an efficient biocatalyst many investigators have constructed recombinant biocatalysts. For example, the *Dsz* genes from *R. erythropolis* DS-3 were successfully integrated into the chromosomes of *Bacillus subtilis* ATCC 21332 and UV1, yielding two recombinant strains, *B. subtilis* M29 and M28, in which the integrated *Dsz* genes were expressed efficiently under control of the promoter Pspac. The DBT desulfurization efficiency of M29 was

16.2 mg DBT l^{-1} h^{-1} at 36 h, significantly higher than that of *R. erythropolis* DS-3 and also showed no product inhibition (Ma *et al.,* 2006b). Tao *et al.,* (2006) constructed a solvent-tolerant desulfurizing bacterium by introducing the gene cluster *DszABCD* from *R. erythropolis* XP into the solvent-tolerant strain *Pseudomonas putida* Idaho. The recombinant strain had the same substrate range as *R. erythropolis* XP and could desulfurize DBT in the presence of *p*-xylene and many other organic solvents at a concentration of 10 % (v/v). Li *et al.,* (2007a) improved the DBT desulfurization activity of *R. erythropolis* DR-1 by removing the gene overlap in the *Dsz* operon. Desulfurization activity of the redesigned strain was about five-fold higher than that of strain DR-1.

An improvement in the uptake of sulfur compounds in oil fractions should be effective in enhancing the biodesulfurization activity. Watanabe *et al.,* (2003) transferred the *Dsz* gene cluster from *R. erythropolis* KA2-5-1 into *R. erythropolis* MC1109, which was unable to desulfurize light gas oil (LGO). Resting cells of the resultant recombinant strain, named MC0203, decreased the sulfur concentration of LGO from 120 mg l^{-1} to 70 mg l^{-1} in 2 h. The LGO-desulfurization activity of this strain was about twice that of strain KA2-5-1. Matsui *et al.,* (2001b) introduced *Dsz* genes into *Rhodococcus* sp. T09, a strain capable of desulfurizing benzothiophene (BT). The resulting recombinant strain grew with both DBT and BT as the sole sulfur sources. The recombinant cells desulfurized not only alkylated BTs, but also various C_x-DBTs, producing alkylated hydroxyphenyls as the end products. Coco *et al.,* (2001) used the DNA-shuffling method to generate recombined genes and evolved enzymes. An increase in steady-state DBT sulfone concentration of at least 16-fold was observed in pathways containing shuffled *DszC* genes.

FMN reductase is among the four key enzymes of the 4S pathway. When flavin reductase, FMN reductase or various oxidoreductases were added to the reaction mixture, or overexpressed in recombinant constructs, the desulfurization rate increased (Gray *et al.,* 1996, 1998; McFarland, 1999; Ohshiro *et al.,* 1995; Rambosek *et al.,* 1999; Squires *et al.,* 1998, 1999). The use of the flavoprotein could result in an approximately 100-fold improvement in the rate of reaction compared with a system

where no flavoprotein was added (Squires *et al.,* 1998). Several research groups have focused on overexpression of this enzyme. FMN reductase can be overexpressed in desulfurizing micro-organisms via mutagenesis. The DNA encoding flavin reductase can be transferred into desulfurizing micro-organisms, can be simultaneously or independently transferred into a desired host cell with the DNA encoding the Dsz enzymes, and can be under the control of the same or a different promoter as the DNA encoding the Dsz enzymes (Squires *et al.,* 1999). Matsubara *et al.,* (2001) amplified the flavin reductase gene with primers designed by using *DszD* of *R. erythropolis* IGTS8; the enzyme was overexpressed in *Escherichia coli*. The specific activity in crude extracts of the overexpressing strain was about 275-fold that of wild-type strain, *R. erythropolis* strain D-1.

As mentioned above, a popular strategy in metabolic engineering is to change the host strain for the *Dsz* genes entirely, perhaps to take advantage of another strain's growth properties, physical properties (for mixing and separations), or higher intrinsic metabolic rate. An interesting feature of genetic experiments involving the *Dsz* genes is that the level of desulfurization activity achieved by genetic manipulation appears to be limited by factors that are not yet understood. In order to achieve very high levels of expression of the desulfurization pathway, a better understanding of the host factors that contribute to the functioning of the pathway is needed (Kilbane, 2006). Therefore, in general, it has been preferred to use the original strain for technical (e.g. gene expression and codon usage preferences) and regulatory reasons (Monticello, 1998). In addition, self-cloning is generally regarded as more effective than heterologous recombination, as gene expression and DBT permeation are readily achieved (Hirasawa *et al.,* 2001; Matsui *et al.,* 2001a).

2.7.5 Reductive C–S Cleavage

Reductive biodesulfurization requires a reducing equivalent. The role of the reducing equivalent is to reduce DBT to biphenyl, releasing the sulfur as hydrogen sulfide (Kim *et al*., 1995). *Desulfovibrio* sp. anaerobically degrades dibenzylsulfide, another organosulfur compound, in the presence of molecular hydrogen to toluene, benzyl mercaptane, and hydrogen sulfide. The reducing equivalent can also be provided by

any other source. A bioelectrochemical process (Kim *et al*., 1990) has been developed to deliver electrons through electrochemical cells to *D. desulphuricans* M6 that resulted in the formation of biphenyl and hydrogen sulfide from DBT. This is shown in Figure 2.9. Studies were also done to observe the effect of the presence of hydrocarbons on reductive DBT degradation.

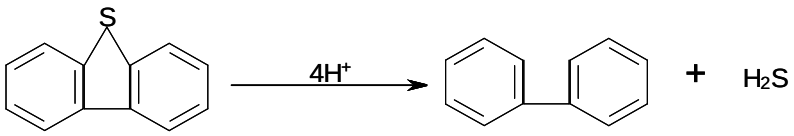


Figure 2.9: The Principle of Sulfur Specific Reductive Pathway of DBT Desulfurization by Bacteria.

When DBT was dissolved in 100% dimethylformamide, an organic solvent (Finnerty, 1993), the products of desulfurization were biphenyl and hydrogen sulfide. However, the hydrogen sulfide production was not solely related to the desulfurization of organosulfur compounds. *Desulfomicrobium longreachii* and *Desulfomicrobium escambium* could degrade only 10% DBT in the presence of 10% v/v kerosene (Yamada *et al*., 2001). Five unknown products were observed, the pathway being reported as different from the one in which biphenyl is the end-product. There are few reports on the desulfurization activity of sulfur-reducing bacteria on DBT and petroleum fractions under well controlled sulfate-reducing anaerobic conditions (Armstrong *et al*., 1995, 1997). No significant reduction in the sulfur content of DBT or in the total sulfur content of vacuum gas oil, deasphalted oil or bitumen was observed. Presently, there is no commercially significant anaerobic biodesulfurization process because of the cost of the hydrogen required and the difficulties in maintaining anaerobic conditions. However, the advantage of this process is that the absence of oxygen prevents the non-specific oxidation of hydrocarbons to colored, acidic, or gum-forming products.

2.7.6 Comparison of Aerobic and Anaerobic Biodesulfurization

The anaerobic route is a potentially attractive biodesulfurization route to apply because of its sulfur specificity. From the pathway, it follows that the calorific value is maintained because C – C bonds are not altered. Furthermore, the reaction pattern is similar to HDS. However, growth under anaerobic conditions proceeds slowly, especially when organic compounds like thiophene and dibenzothiophene are involved in the conversion. From a process point of view, the aerobic route has a major drawback. Sulfur is used as the assimilatory metabolism of aerobic bacteria. Considering that the sulfur content of a biomass is approximately 0.03 wt%, the yield of biomass per mole sulfur removed in the aerobic route is high. Approximately 50% of the energy produced by aerobic microorganisms will be used for growth, while anaerobic microorganisms use approximately 10% of their energy for assimilation. At high biomass concentration, downstream processing is complicated, because proteins originating from the biomass emulsify the oil/water mixture. In addition, mixing efficiency and O_2 availability is less optimal in emulsions with high biomass concentration. Furthermore, diluted sulfate is formed as the end product of the aerobic route that must also be removed, while the H_2S that is formed in anaerobic route can be treated with existing refinery desulfurization plants (e.g. Claus process). In the aerobic sulfur specific route oxygen molecules are added to the hydrocarbon skeleton. This is not desirable, because 2-hydroxybiphenyl is involved in the formation of viscous oil sludge (gum) in the fuel. Furthermore, in product inhibition, 2-hydroxybiphenyl formed in the cells will eventually diffuse back to the oil phase, but the phenolic molecule is a well known biocide.

Based on the aforementioned considerations the anaerobic route is chosen in this thesis.

2.7.7 Commercialization of Biodesulfurization

Most of the published data on biodesulfurization deal with biocatalyst characterization and improvement by classic microbiological methods and genetic engineering. There are very few reports (Monticello, 2000) on biodesulfurization process designs and cost analysis. In order to obtain a biodesulfurization process competitive with the

chemical physical method of hydrodesulfurization, a biotechnological process has to follow three main refinery steps:

1. Separation, entailing some pretreatment of crude oil
2. Conversion i.e. biocatalytic transformation where the biocatalyst favors a selective desulfurization process without destroying useful products
3. Finishing, in which crude oil is separated from the biocatalyst and by-products

Conversion and finishing are affected by a number of crucial factors (viz., biocatalyst specificity, biocatalyst stability, biocatalyst activity, bioreactor design), among which the volumetric ratio between the oil phase and the aqueous medium represents the main limiting factor for an industrial application of the biotechnological process. As described before, microorganisms employ either destructive or non-destructive degradative pathways. Destructive desulfurization is characterized by the cleavage of the C–C bond, leading to a decrease in the fuel value. Thus, this process is unacceptable and cannot be considered for commercial application. In contrast, the non-destructive pathway is advantageous because it specifically removes sulfur without affecting the fuel value. Non-destructive degradation can be either reductive or oxidative. The reductive mechanism is advantageous because the by-products produced are similar to those produced during hydrodesulfurization. Thus, refineries are well equipped to handle them. Moreover, the absence of oxygen checks contamination by other contaminating microorganisms, thus increasing specificity (Monticello and Finnerty, 1985). However, at present there is no commercially significant anaerobic biodesulfurization process because of the hydrogen requirement cost besides the difficulties in maintaining anaerobic conditions. Nevertheless reducing microorganisms are not capable of degrading all sulfur aromatic compounds, in particular the DBT derivatives that are found abundantly in crude and heavy oils. In contrast, oxidative degradation besides not requiring a huge infrastructure as compared with reductive degradation can degrade a large number of sulfur-aromatic compounds. Moreover, the hydrophobic nature of *R. rhodochrous* IGTS8 makes it adhere preferentially to the oil–water interface in oil–water systems (Monticello, 2000). This property is advantageous, as the substrates are directly transferred from the oil into the cell, avoiding mass transfer limitations at this stage.

However, this property makes it difficult to separate cells from oil at a high cell concentration. The difficulty of breaking emulsions by centrifugation led to the invention of a new separation scheme based on filters and more recently hydro cyclones (Chen and Monticello, 1996; Yu *et al*., 1998). Energy Biosystem Corp. (EBC; currently known as Enchira Biotechnology Corp., Tex.), a United States based company, has developed a continuous process of desulfurization of diesel using genetic-recombinant strains of *R. rhodochrous* IGTS8. In partnership with five other organizations they have successfully scaled-up the process to a pilot plant (5 barrels day−1) that has been operating since 1995. Technology has been transferred to an Alaskan refinery, Petrostar for commercial operation, but the scale up of this technology is problematic. Biodesulfurization involves a multi-step metabolic pathway. Thus, whole cells rather than isolated enzyme reactions are needed, moreover, cell-free extracts exhibit a lower activity, in the order of 0.01 g of DBT removed g−1 protein h−1, as compared with 0.4 g/g (biomass h^{-1}) using aerobic microorganisms and 0.1 g/g (biomass h^{-1}) using anaerobic microorganisms (Setti *et al*., 1997). Metabolic pathways due to the presence of other enzymes in the whole cell can be minimized or eliminated by repression of the enzymatic activity, mutation of the cells or denaturation of the undesired enzymes. The research reported in the area of bioreactor design has employed stirred-tank reactors, air-lift reactors, emulsion- phase contactors with free cells, and fluidized-bed reactors with immobilized cells (McFarland *et al*., 1998). Whole-cell immobilization reactors offer advantages as compared to the conventional continuous stirred-tank bioreactors like biocatalyst recovery, increasing the oil/ water volumetric ratio to 90% with respect to the 30–50% of a continuous stirred-tank bioreactor, disposal of a large amount of exhausted medium after treatment, easy separation of oil from biocatalyst and lastly it offers a low risk of microbial pollution. Cell immobilization was recently carried out for the desulfurization of heterocyclic aromatic sulfur compounds by adsorption (Chang *et al*., 2000) and by entrapment. Microorganisms were adsorbed onto celite beads and the immobilized matrix was used as a biocatalyst for the desulfurization of DBT/n-hexadecane model oil and light gas oil in phosphate buffer. The strains could maintain their desulfurization activity until the last batch studied, although a slow

decrease in desulfurization was observed in the case of Gordona sp. as the number of batch cycles increased. Stored biocatalyst however, retained only 50–70% of the initial desulfurization activity of the bacterial strains. Naito *et al*. (2001) used six different matrices e.g. calcium alginate, agar, photo cross linkable resin prepolymers and urethane prepolymers for the desulfurization of a DBT/n- tetradecane mixture by *R. erythropolis* KA2-5-1 under complete non-aqueous conditions. The matrix had a low distribution coefficient of the desulfurized product 2-HBP that has been reported to inhibit the desulfurization of DBT. Setti *et al*. (1997) employed a cell immobilization technique through an adsorption method employing hydrophilic natural supports. This technique does not limit the hydrocarbon uptake mechanism. In fact, the ideal conditions for hydrocarbon biodegradation can be restored at the interface between the oil phase and the hydrophilic support surface. Immobilization also retains water around the catalyst, which is essential for the biocatalytic function. Hydrophilic supports may however compete with the biocatalyst for the available water in the reaction system, especially when the water is also a reactant in the DBT biodegradation pathway so that complete depletion of water from the reaction system results in no biocatalytic activity. The use of hydrophilic supports permits a process in which a decanter unit downstream of the bioreactor is not required, as the water remains entrapped in the support. This factor represents an advantage in terms of finishing costs and times. The activity of the immobilized cells was significantly (up to ten-fold) lower than that of free cells, being in the order of 0.01–0.04 g g−1 biomass h−1, a rate which depended on the kind of support used. The immobilized cells maintained an operational stability for about 100 h, duration similar to that reported for some cell-free bioconversions in n-alkane. Water bioavailability is one of the main factors that still need to be investigated in order to improve the performance of the immobilized biocatalyst. As of today, such a biocatalyst is still not competitive with the physico-chemical process; and the biocatalyst requires further development for this configuration to be competitive. The majority of work on biodesulfurization was performed for the middle-distillate fractions. These results may serve as a background for the desulfurization of other streams. In gasoline, benzothiophene and thiophenes are important sources of sulfur, in addition to DBT. Microorganisms

capable of selectively desulfurizing benzothiophene have been isolated (Kobayashi *et al*., 2000; Matsui *et al*., 2001); and these are seen as potential candidates for gasoline desulfurization. It is interesting that the benzothiophenes desulfurization pathway of *Gordona* sp. strain 213E presents some homology with the *R. rhodochrous* IGTS8 DBT 4S desulfurization pathway (Gilbert *et al*., 1998). Crude oil is another target for sulfur removal and the Japanese are active in this area, although few results have been published on this subject (Ishii *et al*., 2000; Konishi *et al*., 2000; Naito *et al*., 2001). EBC has proposed several options for the integration of biodesulfurization into refineries for diesel production. There are two options: biodesulfurization downstream to hydrodesulfurization or biodesulfurization in place of hydrodesulfurization. Biodesulfurization could be competitive with hydrodesulfurization only in the case of a concomitant production of hydroxybiphenyl sulfinates using the truncated 4S metabolic pathway and the commercialization of these bio-derived surfactants or starting materials for the synthesis of other useful chemicals (Pacheco *et al*., 1999; Lange and Lin, 2001). Thus, biodesulfurization downstream of hydrodesulfurization is an interesting option.

2.8 The Sulfate-Reducing Bacteria

The sulfate-reducing bacteria (SRB) are strict anaerobes; they perform anaerobic respiration by oxidizing certain organic compounds or hydrogen and often other sulfur compounds to hydrogen sulfide (Ilori *et al*., 1999). They play important role in the degradation of organic matter under anaerobic condition (Iverson and Olson, 1984). They use sulfate among other oxidized sulfur compounds as electron acceptors. These organisms are widely distributed in nature where anoxic conditions prevail and are particularly important in shallow sediments of marine environments where neither organic matter nor sulfate is limiting (Johnson and Wood, 1993). Although SRB are obligate anaerobes, their presence and activity have been demonstrated in apparently oxidized environments because they have a cytochrome system like oxidative organisms. Hardy (1981) also found that cells of marine strains of SRB survived well in aerobic and non-aerobic sea water.

2.8.1 Genus *Desulfobacterium*

The cells are oval to rod shaped or slightly curved and a size dimension of 0.7 – 3 x 1.5 – 2.8 µm. The cells are Gram-negative, motile by single polar flagella or nonmotile and strictly anaerobic. Sulfate and in some cases also sulfite and /or thiosulfate are reduced to H_2S. Nutritionally, *Desulfobacterium* species are very diverse and versatile. Many species use H_2, formate and higher monocarboxylic acids up to C_{16}, lactate or ethanol as electron donors. Several species may use aromatic compounds such as phenyl-substituted organic acids, phenolic compounds or N-heterocyclic compounds. They grow poorly on acetate or propionate. H_2-utilizing species are autotrophic. Desulfoviridin is absent. Growth occurs in simple defined media. Yeast extract usually has no stimulatory effect and may even inhibit growth. Most species require vitamins. The optimum pH range is 6.6 – 7.6 while the optimum temperature range in most cases is 20 – 30^0C and is seldom 30 – 35^0C. *Desulfobacterium* species are most common in anoxic marine or brackish sediments. A number of types have been found in anoxic freshwater sediments.

2.8.2 *Desulfobacterium anilini*

A new, rod-shaped, Gram-negative, non-sporing sulfate reducer (strain Anil) was enriched and isolated from marine sediment with aniline as sole electron donor and carbon source was created by Schnell *et al*. (1989). The strain degraded aniline completely to $C0_2$ and NH_3 with stoichiometric reduction of sulfate to sulfide. Strain Anilini also degraded aminobenzoates and further aromatic and aliphatic compounds. The strain grew in sulfide reduced mineral medium supplemented only with vitamin B12 and thiamine. Cells contained cytochromes, carbon monoxide dehydrogenase, and sulfite reductase but no desulfoviridin.

Aniline and aniline derivatives are used on a large scale as starting materials in the manufacture of dyes, pesticides, plastics, and other industrial products. Strain Anil is the first obligate anaerobic bacterium in pure culture growing with aniline as sole electron donor and carbon source. It oxidizes aniline completely to Carbon(IV)oxide and releases the amino nitrogen quantitatively as ammonia.

The most striking feature, however, which clearly differentiates strain Anil from known sulfate-reducing bacteria is its ability to degrade aniline. Therefore, strain Anil is proposed as type strain of a new species, *Desulfobacterium anilini.*

Cells are rod-shaped, 1.25 µm wide and 1.5-3 µm long, Gram-negative, non-sporing, non-motile. Chemoorganotrophic, sulfate or sulfite serve as electron acceptors, elemental sulfur or nitrate not used. Thiosulfate can be dismutated to sulfate and sulfide, without growth. Substrates used are aniline, phenol, p-cresol, several hydroxy- and aminobenzoates, indolylacetate, formate, acetate, higher fatty acids up to stearate, pyruvate, cyclohexanol, cyclohexanone, and cyclohexane carboxylate. Dicarboxylic acids not used. Most substrates completely oxidized to CO_2 via the CO-dehydrogenase pathway. Additions of vitamin B12 and thiamine as growth factors and dithionite 100-200 µM as reducing agent have been found to be necessary. Growth in freshwater and salt water media have been reported (Schnell, 1989). Optimal salt concentrations have been found to be 14g NaCl/L and 2g $MgCl_2$.6 H_20/L. pH-optimum around 6.9-7.5; optimum temperature at 35^0C.

2.8.3 *Desulfobacterium indolicum*

Desulfobacterium indolicum was created by Bak and Widdel in 1986. Indole (1.5 mmol/L) added to sulfate-rich marine mud or sulfate-free sewage digestor sludge was anaerobically degraded within one week. Enrichments from sludge samples in defined indole-containing media with or without sulfate were selective for sulfate-reducing bacteria or mixed methanogenic associations, respectively. Other enrichments of sulfate reducing bacteria were obtained with skatole, indole acetate indole propionate, quinoline, and pyridine. From a marine enrichment: with indole as sole electron donor and carbon source, an oval to rod-shaped, Gram-negative, non spore forming sulfate-reducing bacterium (strain In04) was isolated. Growth occurred in defined bicarbonate-buffered, sulfide-reduced media supplemented with vitamin B Further, aromatic compounds utilized as electron donors and carbon sources were anthranilic acid and quinoline. Nonaromatic compounds used as substrates include formate, acetate, propionate, ethanol, propanol, butanol, pyruvate, fumarate, and succinate. However, growth with substances other than indole was rather slow.

Thiosulfate served as an alternative electron acceptor. Complete oxidation of indole to CO_2 was shown by stoichiometric measurements in batch culture with sulfate as electron acceptor. An average growth yield of 31.3 g of dry cell weight was obtained per mole of indole oxidised. Pigment analysis revealed that cytochromes and menaquinone MK-7 (He) were present. Desulfoviridin could not be detected. Strain In04 was described as new species of the new genus *Desulfobacterium indolicum.*

Strain In04 had oval to rod-shaped cells. The cells bore a single polar flagellum, however, cultures showed only a few motile cells. The dimensions of the cells varied depending of the growth substrate. Cells grown with indole were about 2-2.5 µm in length and 1-1.5 µm in diameter. Strain In04 is Gram-negative.

Absorption spectra of cell-free extracts indicated the presence of b- and c-type cytochromes. Strain In04 contains MK-7 (H2), i.e. a menaquinone with a terminal saturation in the side chain of seven isoprene units. Test for desulfovidridin was negative.

2.9 KINETICS OF BIODEGRADATION

Contamination of the environment with hazardous and toxic chemicals is one of the major problems facing the industrialized nations today. The petroleum industry is responsible for the generation of high amounts of petroleum hydrocarbons and their derivatives as well as for the pollution of air, soils, rivers, seas and underground water. These compounds undergo modifications by either physico-chemical or biological processes. Diverse metabolic capabilities of microorganisms have been exploited by man in diverse ways in the biodegradation of waste materials (Nweke and Okpokwasili, 2006). Microbial activities allowed the mineralization of some petroleum components into Carbon (IV)oxide and water, and microbial transformation is considered a major route for complete degradation of petroleum components (Okpokwasili *et al*., 1986). The potentiality of microbes as agents of degradation of several compounds thus indicates biological treatment as the major promising alternative to attenuate environmental impact caused by pollutants (Nweke and Okpokwasili, 2003)

Knowledge of the kinetics of biodegradation is essential to evaluate the persistence of organic pollutants and to assess the exposure to the environment. In many instances, the information on the kinetics comes only from the evaluation of the loss of parent molecules. In other instances, the knowledge comes from the conversion of organic substrate to inorganic product (Schnell and Turner, 2004). Microbial growth on and utilization of environmental contaminants as substrates have been studied by many researchers. Most times, substrate utilization results in removal of chemical contaminant increase in microbial biomass and subsequent biodegradation of the contaminant. These are all aimed at detoxification of the environmental pollutants. Several microbial growth and biodegradation kinetic models have been developed, proposed and used in bioremediation schemes. Some of these models include Monod's, Andrew's, Bungay's weighted model, General Substrate Inhibition Models (GSIM) and Sum Kinetic Models (Okpokwasili and Nweke, 2006). An understanding of the application of these models and why they may fail to predict the experimental data of biodegradation processes require knowledge of the theoretical bases for these models. The models often used to fit data from evaluation of biodegradation are either empirical or theoretical (Segel, 1975). The study of kinetics of biodegradation in natural environments is often empirical, reflecting the rudimentary level of knowledge about microbial population and activity in these environments. An example of an empirical approach is the power rate model

$$\frac{dC}{dt} = kC^n \qquad 2.1$$

where C is substrate concentration, t is time, k is the rate constant for substrate disappearance and n a fitting parameter. This model can be fitted to substrate concentration versus time curves by varying n and k until a good fit is achieved. From the equation, it is evident that the rate is proportional to a power of the substrate concentration. The power rate law provides a basis for the comparison of different substrate concentration versus time curves, but gives no explanation for the shapes. Therefore, it may have no predictive ability (Schnell and Maini, 2000).

The basic hypothesis of biodegradation kinetics is that substrates are consumed via catalyzed reactions carried out only by the organisms with the requisite enzymes. Therefore, rates of substrate degradation are generally proportional to the catalyst concentration (concentration of organisms able to degrade the substrate) and dependent on substrate concentration characteristic of saturation kinetics (e.g. Michaelis-Menten and Monod kinetics). Saturation kinetics suggests that at low substrate concentrations (relative to the half-saturation constant), rates are approximately proportional to substrate concentration (first order in substrate concentration), while at high substrate concentrations, rates are independent of substrate concentration (zero order in substrate concentration). In the case of substrates that contribute to the growth of the organisms, rates of substrate degradation are linked to rates of growth (i.e. the concentration of the biomass increases with substrate depletion). The mathematical analysis of such growth-linked systems is more complex than those situations where growth can be ignored (Schnell and Maini, 2000). There are a number of situations where it may not be possible to quantify the concentration of substrate-degrading organisms in a heterogeneous microbial community. However, the rate of substrate depletion can be measured. There are also situations in which the organism concentration remains essentially constant even as the substrate is degraded (i.e. no growth situation). Given these various features of biodegradation kinetics, different models including first-order, zero-order, logistic, Monod (with and without growth) and logarithmic models can be used to describe biodegradation (Srere, 1967).

Biodegradation kinetics is used to predict concentrations of chemical substances remaining at a given time during ex situ and in situ bioremediation processes. In most cases, information is based on loss of parent molecule targeted in the process (Segel and Slemrod, 1989). The key interest is frequently the decrease in toxicity concentration. Nevertheless, toxicity measurements require bioassays, which are always very difficult and tedious. Therefore, efficacy of biodegradation is based on chemical measurements, e.g. disappearance of parent molecule, appearance of mineralization products or disappearance of other compounds used stoichiometrically

during biodegradation of a compound, for instance, electron acceptors (Nelson and Cox, 2000). There are several scenarios by which a compound can be transformed biologically. This includes when the compounds serve as:

1. Carbon and energy source
2. Electron acceptor
3. Source of other cell components.

Other scenarios are the transformation of a compound by non-growing cells (the compound does not support growth) and the transformation of a compound by co-metabolism, that is; transformation of a compound by cells growing on other substrate. The simplest case is where the compound serves as source of carbon and energy for the growth of a single bacterial species. The compound is assumed to be water-soluble, non-toxic and other substrates or growth factors are limiting (Stroppolo *et al.*, 2001).

Biochemists usually analyze kinetics parameters within the Michaelis-Menten framework. In particular, the reversible reaction between enzyme E and substrate S, giving the enzyme-substrate complex ES, which irreversibly yields product P has been extensively studied and resulted in the Michaelis-Menten equation,

$$r = \frac{k.C}{K_M + C} \qquad 2.2$$

where r is the rate of the substrate consumption and S is the substrate concentration. A simple expression for an enzyme-catalyzed reaction can be illustrated as shown below

$$E + S \rightleftharpoons ES \longrightarrow E + P$$

The Michaelis-Menten equation allows one to estimate the reaction parameters namely k which is the maximum velocity of the reaction, K_M the Michaelis-Menten constant. The Michaelis-Menten equation assumes a single-enzyme single-substrate system; it does not consider the various steps involved in the transformation of the substrate to the product assume the substrate to be soluble and thus readily available to the organism (Grima, 2007).

Kinetic equations, which describe the activity of an enzyme or a microorganism on a particular substrate, are crucial in understanding many phenomena in biotechnological processes. Quantitative experimental data is required for the design and optimization of biological transformation processes. A variety of mathematical models have been proposed to describe the dynamics of metabolism of compounds exposed to pure cultures of microorganisms or microbial populations of natural environment (Minton, 2001). Characterization of the enzyme or microbe-substrate interactions involves estimation of several parameters in the kinetic models from experimental data. In order to describe the true behavior of the system, it is important to obtain accurate estimates of the kinetic parameters in these models (Olsen, 2006).

Both derivative and integrated forms of equations derived for enzyme catalyzed reactions have been used to estimate kinetic parameters of microbiological processes. Estimates of kinetic parameters K and K_M have been calculated by fitting data to either integrated (Gouder and Delvin, 2001) or derivative (Acuña-Argüelles *et al.,* 2003) forms of Michaelis-Menten and Monod equation. Different approaches have been proposed for estimating the kinetic parameters, but progress curve analysis is the most popular because substrate depletion or product formation data from a single experiment are enough for parameter estimation (Duggleby and Wood, 1989; Zimmerle and Frieden, 1989). In this approach, substrate depletion or product formation-time course is used in the integrated form of the kinetic model for parameter estimation. Some of these differential and integral equations can be found in the papers of Gouder and Delvin (2000).

It is important to note that most kinetic models and their integrated forms are nonlinear. This makes parameter estimation relatively difficult (Zhou *et al.*, 2008). However, some of these models can be linearized. Various linearized forms of the integrated expressions have been used for parameter estimation. However, the use of linearized expression is limited because it transforms the error associated with the dependent variable making it not to be normally distributed, thus inaccurate parameter estimates (Olsen, 2006). Therefore, nonlinear least-squares regression is often used to estimate kinetic parameters from nonlinear expressions. However, the

application of nonlinear least-squares regression to the integrated forms of the kinetic expressions is complicated. This problem and solutions were discussed by Goudar and Delvin (2001). The parameter estimates obtained from the linearized kinetic expressions can be used as initial estimates in the iterative nonlinear least-squares regression using the Levenberg-Marquardt method (Marquardt, 1963; Susu, 1997).

In this study, the two forms of biodesulfurization kinetics were investigated, kinetics with and without mass transfer.

CHAPTER 3

MATERIALS AND METHODS

3.1 STERILIZATION AND ASEPTIC TECHNIQUES

3.1.1 Glassware

All glassware used for the experiments such as beakers, conical flasks etc were washed thoroughly in detergent solution, rinsed in clean tap water and allowed to air-dry before placing them in an oven. The sterilization temperature in the oven was 170^0C for a minimum of three hours.

3.1.2 Work Bench and Incubators

The work bench was always thoroughly disinfected with cotton wool soaked in absolute alcohol. The Bunsen burner was put on to flame the necks of conical flasks and wire loops. The incubators were periodically fumigated with a mixture of 5% phenol and absolute ethanol to reduce the incidence of contamination during incubation.

3.1.3 Growth Media

The growth media used was Nutrient Agar and was prepared according to the Manufacturers' directives and sterilized in an autoclave at a temperature of 121^0C for 15 minutes. Thereafter, the media were allowed to cool to about 45^0C before pouring into sterile Petri dishes. Approximately 15 ml of the medium was aseptically poured into the plates. The medium in the plates was allowed to solidify before inoculation.

3.1.4 Preparation of Agar Slants

Appropriate volumes of the suspended agar were poured into McCartney bottles and sterilized in the autoclave at 121^0C for 15 minutes. After, which they were placed on the laboratory work bench at an angle of 45^0C on the bar and left to solidify.

3.1.5 Inoculating Loop

Inoculating loop was sterilized by flaming the platinum loop over a Bunsen flame until red hot and then allowed to cool before use.

3.1.6 Filter Papers

Filter papers were wrapped in aluminium foil and sterilized by autoclaving at a pressure of 1.1 kg/cm^2 for 15 minutes.

3.1.7 Glass rod

Sterilization of glass rod (hockey stick) was carried out by dipping it in absolute ethanol, igniting over a Bunsen flame and then allowed to cool before use.

3.2 ISOLATION AND IDENTIFICATION OF MICROORGANISMS

3.2.1 Sample Collection

Five samples (about 500g each) of oil contaminated soils were collected randomly at the Orile Petroleum Products Haulage Terminal, Lagos, Nigeria. All the samples were collected in sterile polythene bags. Diesel and kerosene samples (1L each) were obtained from White Dove Filling Station along Badagry Expressway, Ijanikin, Lagos, in new plastic containers. The fuels were obtained from the dispensing machine directly into the containers. The fuels were autoclaved at a temperature of 121^{0}C for 15 minutes in order to destroy organisms that might be present in them.

3.2.2 Isolation of Sulfur-reducing Bacteria (SRB)

In this study, microorganisms with the ability to desulfurize oil were isolated from oil-contaminated soil samples. The medium for isolation of SRB contained the following substances (g/L): S, 1.000; $Na_2S_2O_3$, 0.500; $(NH_4)_2SO_4$, 0.300; KH_2PO_4, 0.250; $CaCl_2.6H_2O$, 0.200; $FeSO_4$.7H2O, 0.250; KCl, 0.500, $MgSO_4$, 0.200; $Ca(NO_3)_2$, 0.500; Sodium propionate,1.000; agar-agar (Oxoid), 15.000 and distilled water to 1000ml mark(pH, 7.4). Then, 2.0g from each of the five randomly collected soil samples were weighed out after mixing and transferred aseptically into an

Erlenmeyer flask containing 90 ml sterile distilled water to give 10^{-1} ten-fold serial dilution. From this, higher dilutions were made up to 10^{-8}. Then, aliquots (0.1ml) from both low (10^{-2}; 10^{-3}; 10^{-4}) and high (10^{-6}; 10^{-8}) dilutions were plated out on the SRB medium in two replicates by spread plate technique using a sterile hockey stick (Collins and Lyne, 1976). The plates were all incubated anaerobically in an anaerobic jar at room temperature ($30^{o}C\pm 2$) for 10-14 days (Frobisher *et al*., 1974; Postgate, 1984). At the end of the incubation, the colonies that developed in the plates were counted; their relative abundance (population density) was estimated by multiplying the plate count by the dilution factor used (Postgate, 1984). Then, pure cultures of the isolates were obtained by streaking technique on SRB medium and stored on agar slants in a refrigerator at $4^{o}C$ when not in use.

3.2.3 Gram Reaction

Gram staining (or Gram's Method) is an empirical method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls. Gram staining is a common procedure in the traditional bacteriological laboratory as a first step to determine the identity of a particular bacterium sample. Thus, Gram reaction helps in the identification of bacterial isolates and was therefore carried out on the sulfur-reducing isolates.

In this study, a thin smear of each pure isolate was prepared on a clean grease-free slide. It was air-dried and heat-fixed by passing it horizontally over a Bunsen flame two or three times. Then, the smear was flooded with crystal violet stain for one minute and rinsed with sterile distilled water. Lugol's iodine was then poured onto the smear for one minute before washing gently with sterile distilled water. The iodine is a mordant; it fixes the crystal violet stain firmly into the cell (Collins and Lyne, 1976). The smear was then decolorized with 70% ethanol continuously until no more crystal violet was running off the slide and immediately rinsed with water. The smear was counter-stained with safranin for 20-30 seconds and then rinsed with water. The slide was blotted dry with a piece of filter paper and finally allowed to air-dry. It was examined under oil immersion objective of the light microscope (Davies *et al*., 2007).

The shape, arrangement and Gram reaction of the cells were observed and recorded.

Staining mechanism

Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall), which stain purple and Gram-negative bacteria have a thinner layer (10% of cell wall), which stain pink. Gram-negative bacteria also have an additional outer membrane which contains lipids, and is separated from the cell wall by the periplasmic space. There are four basic steps of the Gram stain, which include applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture, followed by the addition of a *mordant* (Gram's iodine), rapid decolorization with alcohol or acetone, and counter-staining with safranin or basic fuchsin.

Crystal violet (CV) dissociates in aqueous solutions into CV^+ and chloride (Cl^-) ions. These ions penetrate through the cell wall and cell membranes of both Gram-positive and Gram-negative cells. The CV^+ ion interacts with negatively charged components of bacterial cells and stains the cells purple. Iodine (I^- or I_3^-) interacts with CV^+ and forms large complexes of crystal violet and iodine (CV – I) within the inner and outer layers of the cell. When a decolorizer such as alcohol or acetone is added, it interacts with the lipids of the cell membrane. A Gram-negative cell will lose its outer membrane and the peptidoglycan layer is left exposed. The CV – I complexes are washed from the Gram-negative cell along with the outer membrane. In contrast, a Gram-positive cell becomes dehydrated from an ethanol treatment. The large CV – I complexes become trapped within the Gram-positive cell due to the multi-layered nature of its peptidoglycan. The decolorization step is critical and must be timed correctly; the crystal violet stain will be removed from both Gram-positive and negative cells if the decolorizing agent is left on too long (a matter of seconds).

After decolorization, the Gram-positive cell remains purple and the Gram-negative cell loses its purple color. Counter-stain, which is usually positively-charged safranin or basic fuchsin, is applied last to give decolorized Gram-negative bacteria a pink or red color (Beveridge *et al.*, 2007 and Davies *et al.*, 2007).

Some bacteria, after staining with the Gram stain, yield a *Gram-variable* pattern: a mix of pink and purple cells is seen. The genera *Actinomyces*, Arthobacter, *Corynebacterium*, *Mycobacterium* and *Propionibacterium* have cell walls particularly sensitive to breakage during cell division, resulting in Gram-negative staining of these Gram-positive cells. In cultures of *Bacillus*, *Butyrivibrio*, and *Clostridium* a decrease in peptidoglycan thickness during growth coincides with an increase in the number of cells that stain Gram-negative (Beveridge, 2007). In addition, in all bacteria stained using the Gram stain, the age of the culture may influence the results of the stain. Thus after staining as described above, the bacterial isolates were differentiated and scored Gram positive if they appeared blue (violet) in color under light microscope and Gram-negative if they appear red (pink) (Collins and Lyne, 1976).

Crystal violet is 2 g of 90% crystal violet dissolved in 20 ml of 95% ethanol, Gram's iodine is 1 g of iodine, 2 g of potassium iodide, dissolved in 300 ml of distilled water and decolorizer is 50% ethyl alcohol, 50% acetone (*Bergey et al., 1994)*.

3.2.4 Motility Test

This test is used to determine if an organism is motile or non-motile by means of flagellum. Non-motile bacteria do not possess flagellum.

Motility test was carried out using nutrient agar deep contained in test tube and also by the hanging drop technique.

Agar Dip:

A semi-solid nutrient agar medium was prepared and two day old cultures of each of the two selected isolates were dispensed into test tubes. The nutrient agar in the tubes was allowed to solidify. A suspension of the organism to be tested was prepared in sterile distilled water. A sterile inoculating needle was dipped in the bacterial suspension and used to stab the agar deep straight into the middle of the medium. The process was repeated for all the isolates. The tubes were incubated at 37^0C for two days. At the end of the incubation period, the growth patterns of the isolates were observed. If the growth was confined to the line of stab culture inoculation, the isolate was non-motile. However, if growth of the isolate spread out

into the agar deep away from the line of inoculation, the isolate was motile and therefore had flagella (Benson, 1990).

The Hanging Drop Technique:

A slide with a groove or depression at the centre is used for this test. A drop of the suspension of the isolate was placed inside the groove using a sterile wire loop. With a sterile pin, a small amount of petroleum jelly (Vaseline) was placed at the four edges of a cover slip and used to cover the drop of the bacterial suspension. The slide was observed under the light microscope using oil immersion objective lens. If the isolate displayed a darting motion, it was motile. However, if it did not and flowed in one direction as in Brownian movement, then it is non-motile (Madigan *et al*., 2003).

3.2.5 Catalase Test

Catalase test is dependent on the presence of an enzyme catalase which breaks down hydrogen peroxide, releasing oxygen (MacFaddin, 2000).

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

For this test, a suspension of the organism was made on a clean glass slide and a few drops of 10% v/v hydrogen peroxide added. Effervescence or bubbles of gas in the culture indicated a positive reaction and presence of catalase (MacFaddin, 2000). The test was repeated for all the bacterial isolates. For the negative reaction, no visible reaction was observed

3.2.6 Sugar Fermentation Test

This is a test to determine whether an organism can ferment glucose or any other sugar. Fermentation of glucose results in the abundant production of acidic end products, the presence of which can be detected by the pH indicator in the medium.

Many organisms produce gas – either CO_2 alone or a mixture of H_2 and CO_2. H_2 is insoluble and CO_2 is only slightly soluble and is detected by bubble formation in a Durham tube placed in the medium.

Ten milliliters each of various sugar-indicator peptone water bases with phenol red indicator in test tubes were sterilized by autoclaving at a temperature of 121^0C and a

pressure of 1.1 kg/cm^2 for 15 minutes. The various sugars used for the test were 1% each of glucose, xylose, mannitol, maltose and lactose. The test tubes were inoculated with the isolates and incubated at 37^{0}C for 48 hours. Durham tubes were also placed in the medium for presence of gas (Madigan *et al*., 2003).

3.2.7 The MRVP (Methyl Red Voges Proskauer) Test

MR-VP medium/broth is two tests in one, the Methyl Red Test and the Voges-Proskauer test. MR-VP is a buffered peptone-glucose broth. Organisms that ferment dextrose will release acid into the broth. Adding methyl red, an indicator dye which turns red at pH 4.4 and yellow at pH 6.2, to the inoculated MR-VP medium indicates if the bacteria fermented dextrose. Some bacteria can be distinguished on the basis of their production of acetoin, a neutral end product, after incubation in buffered peptone-glucose media. The addition of alpha-napthol and KOH solutions will result in a pink-red color within a few minutes.

Test Procedure: Culture was inoculated and thereafter incubated at 35^{0}C for 48 hours. Thereafter, aliquots are separated into batches of 5 ml for the methyl red test and the Voges-Proskauer test. This is done in order not to contaminate the original broth tube in case there is need to do further incubation.

(i) Methyl Red Test - About 5 drops of methyl red was added to one of tubes. A red color at the surface is considered a positive result. A negative test is indicated by a yellow color at the surface.

(ii) Voges-Proskaeur Test

The reaction tests for the production of acetyl methyl carbinol (acetoin) from dextrose. This acetoin is oxidized by the reagent to diacetyl which produces a red color with a constituent of the peptone medium.

The dispensed medium was incubated at 37^{0}C for two days. Thereafter, 3ml of 5% alcoholic α-naphtol and 1 ml of 40% potassium hydroxide (KOH) were added and tubes were left for five minutes. A positive reaction was indicated by the development of pink color (Cappuccino and Sherman, 2001). There is no visible change for the negative reaction.

3.2.8 Citrate Utilization Test

The citrate test is used to determine the ability of a bacterium to utilize citrate as its only source of carbon. Bacteria can break the conjugate base salt of citrate into organic acids and Carbon (IV) oxide. The Carbon (IV) oxide can combine with the sodium from the conjugate base salt to form a basic compound, sodium carbonate. A pH indicator in the medium detects the presence of this compound by turning blue (a positive test). For a negative result, the green color of the medium is retained.

Citrate is used by many microorganisms as a sole source of carbon. Cultures were inoculated into slants of Simmons citrate agar and thereafter incubated at 37^0C for 72 hours.

3.2.9 Hydrogen Sulfide Test

Hydrogen sulfide can be produced in small amounts from sulfur-containing amino acids like cysteine by a large number of bacteria in carbohydrate media. Hydrogen sulfide produces on contact with lead acetate a black precipitate, indicated by a visible black-colored reaction on the hydrogen sulfide paper strip. The lead acetate procedure is more sensitive than any other method for detecting hydrogen sulfide production and was used in this study.

Formation of black patches on the lead acetate paper indicated the production of lead sulfide as a result of H_2S production. The lead acetate paper remain unchanged for a negative reaction.

3.2.10 Indole Test

The indole test is a biochemical test performed on bacterial species to determine the ability of the organism to split indole from the amino acid, tryptophan. Like many biochemical tests on bacteria, results of an indole test are indicated by a change in color following a reaction with an added reagent.

Pure bacterial culture must be grown in sterile tryptophan or peptone broth for 24-48 hours before performing the test. To the culture broth, 5 drops of Kovac's reagent (isoamyl alcohol, *p*-Dimethylaminobenzaldehyde, concentrated hydrochloric acid) to the culture broth were added at 37^0C

A positive result is shown by the presence of a red or red-violet color on the surface alcohol layer of the broth. A negative result appears yellow. A variable result can also occur, showing an orange color. This is due to the presence of skatole, also known as methyl indole or methylated indole, another possible product of tryptophan degradation. Isolated cultures were inoculated into peptone water medium and incubated at 37^0C for 48 hours after which 5 drops of Kovac's reagent were added to the culture.

A rose pink colour will indicate the production of indole. A negative result appears yellow

3.2.11. Urease Test

Urease test is a test for differentiating microorganisms. The basis of the test is the ability of the microorganisms to secrete the urease enzyme, which catalyzes the conversion of urea to ammonia and bicarbonate. The urease produced by the microorganisms hydrolyzes urea to ammonia, which raises the pH of the medium, and changes the color of the specimen.

Cultures were inoculated into slants of (Motility, Indole and Urease) MIU medium. MIU mediumwas prepared by adding 0.75g of agar-agar to 100 ml peptone water. Three drops of phenol red indicator was added to the mixture and then autoclaved at a temperature of 121^0C and a pressure of 1.1 kg/cm^2 for 15 minutes. After cooling, 5 ml of 40% urea was added to the mixture. It was then dispensed in sterile bottle, slanted and allowed to set. The culture was then inoculated on the slant. A change in color indicates a positive reaction while the color remained unchanged for the negative test.

3.3 DESULFURIZATION EXPERIMENT

One ml of each of diesel and kerosene was separately mixed with 9 ml of sulfur-free phosphate buffer of pH 7 (prepared as described below) containing 0.5ml of the cells suspension in sterile distilled water (OD_{510}, 0.93) and 2%w/v glucose solution in a 100ml conical flask. The corresponding biomass of cells in the inoculum was approximately 6.40 x 10^6 cfu/ml or 3.20 x 10^6 cfu for the 0.5ml actually inoculated into

the experimental flasks. The flask was then immediately placed into a Edmund Bühler Johanna otto GmbH shaker incubator for 12 hours. The temperature of the shaker was 30^0C and operated at a speed of 180 rotations per minute (rpm). The experiments were also conducted for 24, 36, 48, 60 and 72 hours in duplicates. The microbial activity on the fuel was stopped with 1ml 0.1M sodium hydroxide (NaOH) added to the flasks after 1ml was withdrawn for microbiological analysis. Thereafter, the separation of the oil and cell suspension was done by centrifugation at a rotational speed of 5,000 x g for 30 minutes. To determine the total sulfur content of the oil, the oil phase was removed with a syringe and injected directly into a GC-PFPD (Chang *et al.,*, 2000). A control design was also set up just like the experimental design but without inoculation with the cell suspension.

3.3.1 Phosphate Buffer of pH Value of 7

This was prepared by weighing 0.5g Na_2HPO_4 (disodium hydrogen phosphate) and 3.0g KH_2PO_4 (potassium dihydrogenphosphate). After weighing, the two substances were placed into a measuring cylinder containing 500 cm^3 distilled water. Then, the cylinder was made up to 1000 cm^3 mark by adding more distilled water. The pH of the prepared buffer solution was standardized using a pH meter. The buffer solution was then sterilized in an autoclave at a temperature of 121^0C for 15 minutes before it was used for the experiments as described above.

3.3.2 Analytical Methods for Determining Sulfur Content

The optical density (OD) of the cells of the isolated microorganisms used for the experiments was measured turbidimetrically at 510 nm as a function of growth using Sherwood model colorimeter. The colorless phosphate buffer was used as the blank for the colorimeter to set its absorbance at zero after which the OD of the cell solution was determined. The concentrations of various sulfur compounds were determined by gas chromatography with Pulsed Flamed Photometric Detector (GC-PFPD, type 5890A; Hewlett Packard, Mississauga, USA).

The GC was equipped with a glass column (3.2mm x 1m) that had been packed with silicone and 2% chromosorb. The flow rate of the nitrogen carrier gas was 15 ml/min. the column temperature was programmed from 120^0C through 250^0C at 5^0C/min. The injector and detector temperature was maintained at 250^0C and 350^0C respectively. For the detection of sulfur-containing organic compounds, a sulfur-selective Pulsed Flame Photometric Detector connected to the capillary column was used (Chang *et al.,* 2000). Sulfur-containing organic compounds: namely; thiophene, 2, 5 – dimethyl thiophene, benzothiophene and dibenzothiophene were run as standards and used to identify the peaks in diesel and kerosene samples for both the control and experimental designs. Then the changes in concentration of these sulfur-containing compounds in diesel and kerosene with time were extrapolated. The values were plotted against time.

3.4 KINETICS OF ENZYME CATALYZED REACTIONS

3.4.1 Steady- State Approximation

In the derivation of kinetic equations for homogeneous and heterogeneous catalytic reactions, it is necessary to use some approximation in order to reduce the derived equation to manageable forms. Chemical reactions occur through some reactive intermediates. These intermediates can be conceived as energized reactants with the requisite energy to pass into the final state and form a product. These reactive intermediates could be atoms or free radicals. In heterogeneous catalysis, these intermediates adsorb species with mobility to search for energetically favorable sites for subsequent conversion and desorption. In order to derive the rate of a chemical reaction, the various elementary reactions are tabulated and appropriate differential equations are written for individual reactions. The concentrations of the intermediates are then eliminated and the resulting equations are solved to obtain the rate of reaction (Susu, 1997).

The steady state approximation allows a simple procedure where the intermediates, whose concentrations are low, are assumed to have a constant concentration during the course of the reaction. By this approximation, simple expressions for the

concentration of intermediates are derived and hence the overall reaction rate determined.

A simple enzyme-catalyzed reaction can be illustrated as shown below

$\underline{E} + S \rightleftharpoons \underline{ES}$ 3.1

$\underline{ES} \rightleftharpoons P + \underline{E}$ 3.2

E denotes the enzyme that is a catalyst and thus provides the active site(s) on which the reaction will take place while S is the substrate and its concentration C. All the surface species are underlined. The forward rate of the ith step is denoted by k_i while that of the reverse step is k_{-i} .

Using the method outlined by Boudart (Susu, 1997),

$a_1 = k_1(A)$ 3.3

$a_{-1} = k_{-1}(A)$ 3.4

where the 'a's are the pseudo first order rate constants.

The net rate of each step r_i according to the steady state approximation.

$r_1 = r_2 = r$ 3.5

thus, the two algebraic equations necessary for the solution of E and ES are

$a_1E - a_{-1}ES = r$ 3.6

$-a_{-2}E + a_2ES = r$ 3.7

or

$$\frac{a_1E}{r} - \frac{a_{-1}ES}{r} = 1 \quad 3.8$$

$$\frac{-a_{-2}E}{r} + \frac{a_2ES}{r} = 1 \quad 3.9$$

The unknowns are $\frac{E}{r} and \frac{ES}{r}$ can be solved by applying Cramer's rule to the following sets of rearranged equations

$a_1E_1 - a_{-1}E_2 = 1$ 3.10

$-a_{-2}E_1 + a_2E_2 = 1$ 3.11

where

$E_1 = \frac{E}{r}$ and $E_2 = \frac{ES}{r}$,

The solution is given by $E_i = \frac{N_i}{D}$ i = 1, 2 3.12

where N_i and D are determinants.

$$D = \begin{bmatrix} a_1 & -a_{-1} \\ -a_{-2} & a_2 \end{bmatrix} \quad N_1 = \begin{bmatrix} 1 & -a_{-1} \\ 1 & a_2 \end{bmatrix} \quad N_2 = \begin{bmatrix} a_1 & 1 \\ -a_{-2} & 1 \end{bmatrix}$$

Thus.

$D = a_1a_2 - a_{-1}a_{-2}$ 3.13

$N_1 = a_2 + a_{-1}$ 3.14

$N_2 = a_1 + a_{-2}$ 3.15

Therefore,

$$\sum_{i=1}^{2} N_i = a_2 + a_{-1} + a_1 + a_{-2} \quad 3.16$$

If L is the total concentration of active sites on the enzyme surface, then

$L = E + ES$ 3.17

Thus,

$$\frac{L}{r} = \frac{E}{r} + \frac{ES}{r}$$

$$\frac{L}{r} = \frac{N_1}{D} + \frac{N_2}{D}$$

$$\frac{L}{r} = \frac{\sum_i N_i}{D}$$

$$r = L\frac{D}{\sum_i N_i} \quad 3.18$$

The expression for the rate of reaction is

$D = k_1k_2C - k_{-1}k_{-2}P$ 3.19

$$\sum_{i=1}^{2} N_i = k_2` + k_{-1} + k_1C + k_{-2}P \quad 3.20$$

Thus,

$$r = \frac{k_1k_2C - k_{-1}k_{-2}P}{k_2 + k_{-1} + k_1C + k_{-2}P} \quad 3.21$$

In biological reactions, the rate of product formation is generally slow; this is due to the multi-step nature of the conversion of the substrate to the final product. This makes the concentration of the product to be small compared to that of the substrate. Often, in biodegradation the interest has always been in the disappearance of the substrate. For the purpose of this work the product shall be neglected so that C >> P.

$$r = \frac{k_1 k_2 C}{k_2 + k_{-1} + k_1 C} \qquad 3.22$$

$$r = \frac{k_2 C}{\frac{k_2 + k_{-1}}{k_1} + C} \qquad 3.23$$

the ratio of the rate constants can be represented by K_M so that

$K_M = \frac{k_2 + k_{-1}}{k_1}$ the usual form of Michaelis-Menten constant so that equation 3.21 becomes

$$r = \frac{k_2 C}{K_M + C} \qquad 3.24$$

the usual form of the Michaelis-Menten equation.

The method of steady state approximation as outlined by Boudart (Susu, 1997) has successfully been used to obtain the Michaelis-Menten equation. The latter equation shows that enzyme-catalyzed reactions are a special type of heterogeneous catalytic reactions for which product concentration is relative to that of the substrate.

3.4.2 The Rate-Determining Step Method

One formalized way of arriving at the simpler forms of the rate expression is by the use of the concept of the rate-determining step. The concept is simply stated thus 'In a sequence of elementary steps, one step is postulated to be rate determining and all the other steps in the sequence are assumed to be in a quasi-equilibrium.' The consequence of the concept is that only that step is kinetically significant as it is the only step that appears in the rate expression. The rate constants of the other steps appear as the ratios that are equal to the equilibrium constants of those steps (Susu, 1997).

Considering a simple enzyme-catalyzed reaction illustrated as shown below,

E + S ⇌ ES

ES ⇌ P + E

Applying the concept of the rate-determining step to the above illustrated chemical reaction, if step 1 determines the rate of the overall reaction i.e. the rate–determining step, then ES in the second step is in quasi-equilibrium with E and P and vice versa. Then

$a_2ES = a_{-2}E$ 3.25

so that L = ES + E (conservation of active site) 3.26

Hence, $ES = \frac{L}{1+\frac{a_2}{a_{-2}}}$ 3.27 and $E = \frac{L}{1+\frac{a_{-2}}{a_2}}$ 3.28

$r_1 = a_1E - a_{-1}ES$ 3.29

$r_1 = L\left(\frac{a_1a_2}{a_2+a_{-2}} - \frac{a_{-1}a_{-2}}{a_2+a_{-2}}\right)$ 3.30

setting L to unity , then $r_1 = \frac{k_2k_1C - k_{-1}k_{-2}P}{k_2 + k_{-2}P}$ 3.31

Enzyme catalyzed reactions are hardly reversible with respect to the final product and is therefore assumed that the reaction is an irreversible one so that the irreversible term is equal to zero.

$$r = k_1C$$ 3.32

a first order reaction.

If the second step determines the rate of the overall reaction, then

$a_1E = a_{-1}ES$ 3.33

So that, $E = \frac{L}{1+\frac{a_1}{a_{-1}}}$ 3.34 and $ES = \frac{L}{1+\frac{a_{-1}}{a_1}}$ 3.35

$r_2 = a_2ES + a_{-2}E$ 3.36

$r_2 = L\left(\frac{a_1a_2}{a_1+a_{-1}} - \frac{a_{-1}a_{-2}}{a_1+a_{-1}}\right)$ 3.37

setting L to unity , then

$$r_2 = \frac{k_2 k_1 C - k_{-1} k_{-2} P}{k_1 C + k_{-1}} \quad 3.38$$

As earlier mentioned, in biological reactions, the rate of product formation is generally slow; this is due to the multi-step nature of the conversion of the substrate to the final product. This makes the concentration of the product being always small compared to that of the substrate. Often, in biodegradation the interest has always been in the disappearance of the substrate. Again for the purpose of this work the product shall be neglected so that C >> P may be assumed.

$$r_2 = \frac{k_1 k_2 C}{k_{-1} + k_1 C} \quad 3.39$$

$$r_2 = \frac{k_2 C}{\frac{k_{-1}}{k_1} + C} \quad 3.40$$

so that the ratio of the rate constants can be represented by $K_M = \frac{k_{-1}}{k_1}$ the usual form of Michaelis-Menten constant so that equation 3.38 becomes

$$r_2 = \frac{k_2 C}{K_M + C} \quad 3.41$$

the usual form of the Michaelis-Menten equation.

The rate-determining step using the method as outlined by Boudart has also been successfully used to obtain the Michaelis-Menten equation. The method has also pointed to the fact that the step, which determines the overall rate of reaction, is the second step which is the decomposition of the enzyme-substrate complex to the product and enzyme.

3.5 MODEL DEVELOPMENT

3.5.1 Development of the Kinetic Model of Biodesulfurization

The kinetic model of biodesulfurization shall be developed based on the sulfur specific pathways; namely the oxidative pathway popularly known as the 4S pathway and the reductive pathway. The two pathways did not lead to loss of energy value of the fuel (diesel and kerosene).

The mechanism of the 4S pathway involves the sequential oxidation of the sulfur moiety and the cleavage of the carbon – sulfur bonds. This system consists of two monooxygenases, DszC and DszA which sequentially oxidize DBT to DBT sulfone through DBT sulfoxide and 2-hydroxybiphenyl-2'-sulfinic acid, an NADH-flavin mononucleotide oxidoreductase (DszD) which supplies the two monooxygenases with reduced flavin, a desulfinase (DszB) which converts 2-hydroxybiphenyl-2'-sulfinic acid to the desulfurized end product, 2 -hydroxybiphenyl.

This mechanism is similar to a consecutive reaction in which the product of one step becomes the substrate in another step to form the product for the next stage.

This mechanism is therefore a multiple enzymes system with a substrate for a model substrate situation.

$$S \xrightarrow[E1]{r_1} P1 \xrightarrow[E1]{r_2} P2 \xrightarrow[E2]{r_3} P3 \xrightarrow[E3]{r_4} P4$$

S is the model substrate (DBT), E1 is DszC, P1 is DBT sulfoxide, P2 is DBT sulfone, E2 is DszA, P3 is 2-hydroxybiphenyl-2'-sulfinic acid, E3 is DszB and P4 is 2-hydroxybiphenyl. Hence,

$$r(C) = -\frac{dC}{dt} = \frac{k_{21}C}{K_{M1} + C} \tag{3.42}$$

$$r(P_1) = \frac{dP_1}{dt} = \frac{k_{21}C}{K_{M1} + C} - \frac{k_{22}P_1}{K_{M2} + P_1} \tag{3.43}$$

$$r(P_2) = \frac{dP_2}{dt} = \frac{k_{22}P_1}{K_{M2} + P_1} - \frac{k_{23}P_2}{K_{M3} + P_2} \tag{3.44}$$

$$r(P_3) = \frac{dP_3}{dt} = \frac{k_{23}P_2}{K_{M3} + P_2} - \frac{k_{24}P_3}{K_{M4} + P_3} \tag{3.45}$$

$$r(P_4) = \frac{dP_4}{dt} = \frac{k_{24}P_3}{K_{M4} + P_3} \tag{3.46}$$

So that

$$r(S) = r(C) + r(P_1) + r(P_2) + r(P_3) + r(P_4) \quad 3.47$$

$$r(S) = \frac{2k_{21}C}{K_{M1} + C} \quad 3.48$$

Equation 3.48 represents the kinetics of the sulfur specific oxidative (4S) pathway of biodesulfurization most especially when the initial concentration C_0 of the substrate is approximately the Michaelis-Menten constant K_M i.e. ($C_0 \sim K_M$). Therefore, when $C_0 >> K_M$ the kinetics follow zero order and when $C_0 << K_M$ then the kinetics follow the first order.

In the development of kinetics of the sulfur specific reductive pathway of biodesulfurization, the mechanism adopted is that in which DBT is used as the sole electron acceptor and sulfur is removed selectively (Kim *et al*., 1990a). Biphenyl was found as the major reaction product. It is a single step reaction.

$$S \xrightarrow[E]{H} P5 \quad 3.49$$

Where P_5 is the biphenyl and H is hydrogen. Hence, the rate of DBT disappearance,

$$r(C) = -\frac{dC}{dt} = \frac{kC}{K_M + C} \quad 3.50$$

Equation 3.50 represents the kinetics of the sulfur specific reductive pathway of biodesulfurization most especially when the initial concentration C_0 of the substrate is approximately the Michaelis-Menten constant K_M i.e. ($C_0 \sim K_M$). Therefore, when $C_0 >> K_M$ the kinetics follow zero order and when $C_0 << K_M$ then the kinetics follow the first order.

3.5.2 Development of the Mass Transfer Model of Biodesulfurization

According to Goswami (1991) and Monticello (1998), the microorganisms carrying out the biodegradation become attached directly to the NAPL (the organosulfur

compounds) and there, the microorganism secretes the enzymes that will carryout the desulfurization there at the surface to desulfurize the substrate. This is assumed because the size of the substrate (the molecules of DBT and its derivative) are too large for permeability into the cytoplasm. Hence the enzymes are described as being extracellular and the reaction takes place on the surface. In other words, in the case of the sulfur specific oxidative (4S) pathway biodesulfurization, the microorganism desulfurizes the substrate (the multicomponent NAPL) to form various sulfoxides, sulfones, 2'-hydroxybiphenyl-2-sulfinates and the hydroxybiphenyl on the surface.

For the reductive pathway, the DBT was selectively reduced to biphenyl.

A model for the transport process will be developed based on this reasoning.

The first step is to develop a conceptual picture (Figure 3.1) to describe the physical system through substrate and oxygen transport mechanisms and biodesulfurization.

Resistances to mass transfer can be encountered at eight possible locations, namely;

(1) In the gas film;

(2) At the gas/liquid interface;

(3) In the liquid film surrounding the gas interface;

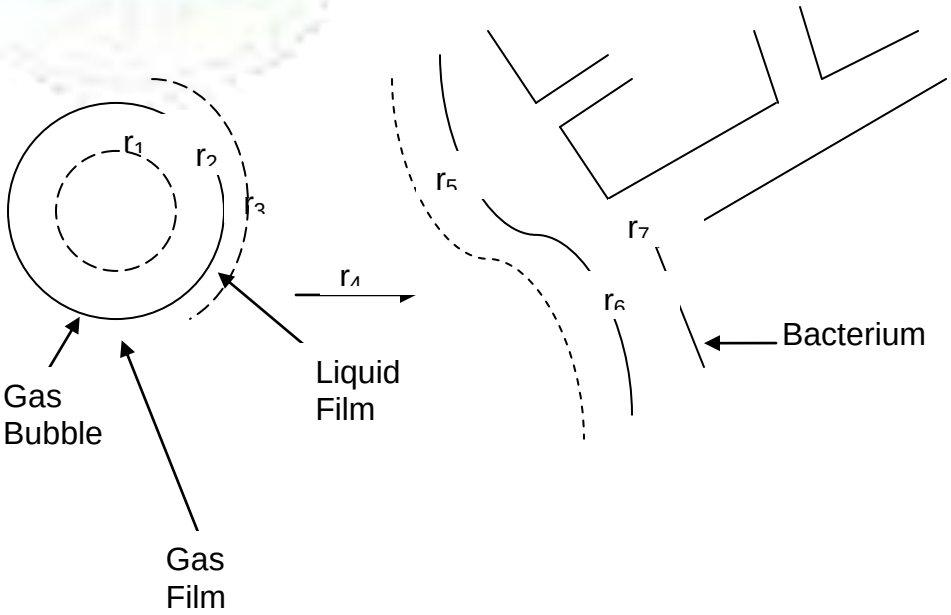


Figure 3.1: The Pathways of Oxygen or Hydrogen (The Gas Phase) and Substrate Transfer to a Microorganism in a Bioreactor

(4) In the liquid phase containing the substrate;

(5) In the liquid film surrounding the solid (microorganism);

(6) At the liquid/solid interface;

(7) The site of reaction; and

(8) In the solid phase.

These resistances occur in series and the largest of them is the most significant and will be rate-controlling. Thus, the entire mass transfer pathway can be modeled using a mass transfer correlation.

Some of these resistances cannot be rate-controlling.

1. Since gas-phase mass diffusitivities are typically much higher than liquid phase diffusitivities, the resistance of the gas film in the gas phase can be neglected relative to the liquid film surrounding the bubble.
2. The interfacial resistance to transport at the gas/liquid interface is negligible.
3. The resistance at the liquid/solid interface can thus be neglected as well.
4. Provided the liquid is well-mixed, transport through the liquid phase is generally rapid and bulk fluid resistance is neglected.
5. The resistance in the solid phase (microorganism) may also be neglected because the size of the substrate (the molecules of DBT and its derivative) is too large for permeability into the cytoplasm. Hence the enzymes are described as being extracellular.

The reaction takes place on the surface of the organism.

Two mass transfer resistances and the reaction rate remain to be considered and these are the two liquid film resistances. Depending on the size of microbial particle, any one of these resistances can be controlling.

1. In the case of small microbial pellets, their very small size and hence large interfacial area relative to that of gas bubble will result in the liquid film surrounding the gas bubble being the rate determining step in oxygen transport.
2. On the other hand, large microbial pellets may be of a size comparable to that of a gas bubble and resistance in the liquid film surrounding the solid (microorganism) may dominate. (For the purpose of this work, this resistance was chosen putting into consideration the size of DBT and its derivative).

The resistance on the surface of the microorganism results from diffusion and reaction of oxygen and the substrate. The overall rate of substrate conversion is governed solely by the kinetics of the reaction. However, if mass transfer is slow relative to reaction, transport may influence the observed kinetic rates. Oxygen acts as an electron acceptor in the sulfur specific oxidative (4S) pathway of biodesulfurization from the substrate during the biodesulfurization reaction. The role of the hydrogen in the reductive process is that of electron donor to the DBT. This implies that the substrate in the bulk liquid will diffuse to the surface of the cell where it is adsorbed and undergoes biodesulfurization.

Mass balance of the substrate in the liquid phase (Fuels) is presented as follows:
The material balance on the solute (substrate in oil) over the time period from t to $t + \Delta t$ over the element of volume of a batch reactor from z to $z + \Delta z$ is obtained thus:
(Input at z) – (output at $z + \Delta z$) – (Reaction due to biodesulfurization) – (Rate of transfer to the cell surface) = Accumulation over time period. 3.51
In a batch reactor, there is no inflow or outflow of material thus,
Accumulation over time period = (Rate of transfer of the substrate to the cell surface) - (Reaction due to biodesulfurization)
Mass transfer rate to the solid, $r_m = ak_L(C - C_S)A\Delta z$ 3.52

Accumulation in the fluid phase, $r_{ac} = A\Delta z\left(\frac{dC}{dt}\right)$ 3.53

There is no reaction in the fluid phase in the reactor since all the reactions take place on the cell surface. Hence the reaction term is equal to zero.
Substituting equations 3.52 and 3.53 into equation 3.51

$$k_L a(C - C_S)A\Delta z = A\Delta z\left(\frac{dC_i}{dt}\right) \quad 3.54$$

Dividing equation 3.54 by $A\Delta z$ we have

$$k_L a(C - C_S) = \frac{dC}{dt} \quad 3.55$$

C_S is the concentration of the substrate on the surface of the organism and is not measurable.

The solution to the problem can be obtained by applying the law of conservation of mass to the adsorbable solute contained in the fluid phase and the solid.

It should be noted that adsorption transfer material from the fluid phase and adds to the solid phase.

The solid phase loses material by desulfurization and generates none.

Then the solid phase mass balance for the sulfur specific oxidative (4S) pathway of biodesulfurization is

$$A\Delta z\frac{dq}{dt} - A\Delta z\frac{2kC}{K_M + C} = k_L a\left(C - C_S\right)A\Delta z \qquad 3.56$$

Dividing Equation 3.56 through by the elementary volume, $A\Delta z$ yields Equation 3.57

$$\frac{dq}{dt} - \frac{2kC}{K_M + C} = k_L a\left(C - C_S\right) \qquad 3.57$$

Substituting Equation 3.57 into equation 3.55, we have

$$\frac{dq}{dt} - \frac{2kC}{K_M + C} = \frac{dC}{dt} \qquad 3.58$$

Solutions to equation 3.58 are simple when q is a linear function of C, that is, the adsorption, is assumed to be linear. Then $\frac{dq}{dt}$ can be replaced by $-K\frac{dC}{dt}$.

Then, $\frac{dq}{dt} = K\frac{dC}{dt}$ 3.59

K is Distribution coefficient

$$-\frac{2kC}{K_M + C} = \frac{dC}{dt} + K\frac{dC}{dt} \qquad 3.60$$

$$\frac{2kC}{K_M + C} = -\left(1 + K\right)\frac{dC}{dt} \qquad 3.61$$

Equation 3.61 represents the mass transfer based kinetic rate expression for the sulfur specific oxidative pathway of biodesulfurization and is a first order differential equation.

Solving equation 3.61 analytically, we integrate taking the appropriate limits.

Therefore:

$$\int_0^t \frac{2k}{(1+K)}dt = -\int_{C_0}^{C}\left(1+\frac{K_M}{C}\right)dC \qquad 3.62$$

On integrating and taking the limits of that [C] = $[C]_0$ at time t = 0:

$$\frac{2k}{(1+K)}t = (C_0 - C) - K_M \ln\frac{C}{C_0} \qquad 3.63$$

Equation 3.63 is clearly an implicit equation.

For the sulfur specific reductive pathway biodesulfurization, equation 3.50 is substituted for the reaction term of equation 3.56. Thus the mass transfer based kinetic rate expression for the sulfur specific reductive pathway of biodesulfurization is given by $\frac{kC}{K_M + C} = -(1+K)\frac{dC}{dt}$ 3.64

Similarly, integrating and taking the limits [C] = $[C]_0$ at time t = 0, equation 3.64 yields

$$\frac{k}{(1+K)}t = (C_0 - C) - K_M \ln\frac{C}{C_0} \qquad 3.65$$

Equation 3.65 is an implicit equation.

It is difficult to generate a substrate concentration – time data from equations 3.63 and 3.65 because of their implicit nature. Such a profile can be obtained by using any of the following techniques:

1. Application of the Finite Difference Method to equations 3.61 and 3.64
2. Application of the Implicit 4th order Runge-Kutta Method to equations 3.61 and 3.64
3. Application of the Newton-Raphson Method to equations 3.63 and 3.65

In this study, the Finite Difference Method was chosen and used to numerically integrate equations 3.50 and 3.64 which represent the kinetics without mass transfer

influence and mass transfer influenced kinetics respectively of the sulfur specific reductive pathway of biodesulfurization of diesel and kerosene.

The enumerated numerical methods are described in details in Section 3.7

3.6 KINETIC PARAMETER ESTIMATION OF BIODESULFURIZATION OF DIESEL AND KEROSENE

3.6.1 Non-Linear Regression Analysis

The kinetic parameter estimation of the biodesulfurization of diesel and kerosene is presented in this section. The experimental data of the biodesulfurization of diesel and kerosene were subjected to kinetic analysis with a view to estimate associated rate parameters.

The rate (r) of enzyme-catalyzed reactions is hyperbolically related to the substrate concentration (C) through the Michaelis-Menten equation:

$$r = \frac{dC}{dt} = \frac{kC}{K_M + C} \qquad 3.66$$

where k and K_M are kinetic constants characteristic of the particular enzyme and substrate. The kinetic equation for biodesulfurization of diesel and kerosene (3.48) is similar to equation 3.66 and these rate constants can be estimated using experimental substrate concentration versus time data. They are usually extracted by using linear transformations such as the Lineweaver-Burk or Hanes plots.

The linear plot equations are as follows:

Hanes Plot:

$$\frac{C}{r} = \frac{K_M}{k} + \frac{C}{k} \qquad 3.67$$

For the Hanes plot, a plot of $\frac{C}{r}$ against C should give a straight line. The reciprocal of the intercept at the ordinate gives the maximum rate k. K_M can then be estimated from the slope.

Lineweaver-Burk Plot:

$$\frac{1}{r} = \frac{K_M}{k}\frac{1}{C} + \frac{1}{k} \qquad 3.64$$

For the Lineweaver-Burk Plot, a plot of $\frac{1}{r}$ against $\frac{1}{C}$ should also give a straight line. Also the reciprocal of the intercept at the ordinate gives the maximum rate k. K_M can also be subsequently estimated from the slope.

Eadie-Hofstee Plot:

$$r = k - \frac{r}{C}K_M \qquad 3.69$$

For the Eadie-Hofstee Plot, a plot of r against $\frac{1}{C}$ should also give a straight line. The maximum rate k is the intercept. Similarly, K_M can then be estimated from the slope.

These transformations are unsatisfactory as they can result in artificial weighting of the data leading to erroneous estimates of k and K_M, to forestall this development, the experimental substrate concentration time data was subjected to non-linear regression analysis.

In linear regression, the $\sum(residuals)^2$ is minimized, where the residual represents the difference between experimental and calculated function. If equation 3.50 is used directly, then the problem of parameter estimation becomes non-linear and the minimization of the sums of residuals becomes iterative.

The iterative method for estimating the kinetic parameters k and K_M using the Marquardt's algorithm of non-linear regression analysis entails that the multi-response nature of the problem will be accounted for by the minimization of the sums of the squares of the residuals Φ on the molar quantities of the reaction (Susu, 1997).

The minimization of the sum of squares of the residuals can be represented by:

$$\Phi = \sum(F_j^0 - F_j)_i^2 \qquad 3.70$$

where i = 1….L (L = number of experiments), j = 1…N (N = number of components), F_j^0 = experimental molar quantity and F_j = predicted molar quantity.

In this procedure, the parametric corrections δ were evaluated by solving the following set of equations (Susu, 1997).

$$(A^TA + \lambda I)\ \delta = A^Te \qquad 3.71$$

where

$$A = \begin{bmatrix} \frac{\partial F_1}{\partial k_1} & \frac{\partial F_1}{\partial k_2} & \frac{\partial F_1}{\partial k_3} \\ \frac{\partial F_2}{\partial k_1} & \frac{\partial F_2}{\partial k_2} & \frac{\partial F_2}{\partial k_3} \\ \frac{\partial F_3}{\partial k_1} & \frac{\partial F_3}{\partial k_2} & \frac{\partial F_3}{\partial k_3} \end{bmatrix}$$

A^T is a transpose of matrix A,

e = vector of differences between experimental and predicted molar quantities;

$$e = \begin{bmatrix} \Delta k_1 \\ . \\ . \\ . \\ \Delta k_m \end{bmatrix} \qquad 3.72$$

λ = an arbitrarily chosen scalar quantity and I is an identity matrix.

$$\delta = \begin{bmatrix} \Delta k_1 \\ \Delta k_2 \\ \Delta k_3 \end{bmatrix} \qquad 3.73$$

I = identity matrix

The partial derivatives $\frac{\partial F_j}{\partial k_m}$ are evaluated by use of:

$$\frac{\partial F_1}{\partial k_1} = \frac{F(k_1,....,k_m) - F_1(k_1 + gk_1,....,k_m)}{gk_1} \qquad 3.74$$

where g takes an arbitrary value suitable for the iteration.

For the algorithm;

1 Some set of parameter values was assumed.

2 The vector residuals e was estimated by use of equation 3.72

3 The elements of matrix A were estimated by use of equation 3.73.

4 A^T was estimated.

5 Equation 3.71 was solved for e for the parametric correction vector, δ.

6 Φ was estimated by the use of equation 3.70

7 Kinetic parameters were upgraded according to equation 3.75:

$$k_m = k_m + \Delta k_m \qquad 3.75$$

8 The whole procedure was repeated until the termination was satisfied

The algorithm was performed on the experimental results obtained from the biodesulfurization of diesel and kerosene.

The latter techniques resulted in good estimates of the rate constants.

3.6.2 Estimating the Partition (Distribution) Coefficient

This is the ratio of concentrations of a compound in two phases of a mixture of two immiscible solvents at equilibrium. Hence, it is a measure of differential solubility of the compound between these two solvent.

Environmental fate assessment of fuel components relies heavily on fuel-water partition coefficient ($K_{i,fw}$) values , defined as

$$K_{i,FW} = \frac{\text{solute concentration in the fuel phase}}{\text{solute concentration in the aqueous phase}} \quad 3.76$$

for solute *i* distributed between a fuel (*f*) phase and an aqueous (*w*) phase. In this work, the model developed by Aren and Gschwend (2005) was used in estimating the partition coefficient ($K_{i,fw}$) values of sulfur-containing organic substances in the fuels.

Raoult's law is frequently used to model nonpolar solutes in fuels and related mixtures (Schwarzenbach *et al*, 2003), In this case, the fuel-water partition coefficient may be expressed as

$$K_{i,fw} = K_{i,fa}K_{i,aw} = \frac{RT}{V_f P^0_{l,i}} K_{i,aw} \quad 3.77$$

where *Ki,fa* is the fuel-air partition coefficient of solute *i*, *Ki,aw* is the air-water partition coefficient of solute *i*, *R* is the molar gas constant, *T* is temperature, *Vf* is the molar volume of the fuel phase, and *P°l,i* is the (hypothetical) liquid vapour pressure of solute *i*. Raoult's law would be inappropriate for modeling the aqueous phase, since nonpolar organic solutes are known to experience significant nonideality in aqueous conditions (Yalkowsky, 1999).

In the model developed by Aren and Gschwend (2005), Raoult's law was applied to the sulfur-containing organic substances in the fuel phase but used an air-water

linear solvation energy relationships (LSERs) (Abraham *et al*, 1994) to treat the aqueous phase.

$$\log K_{i,fw} = \log\left[\frac{RT}{V_f P_i^0}\right] - \left(C_{aw} + r_{aw}R_2 + S_{aw}\Pi_2^H + a_{aw}\alpha_2^H + b_{aw}\beta_2^H + V_{aw}V_x\right) \qquad 3.78$$

The parameters R_2 describes the excess molar refraction of solute i (Abraham *et al*, 1999), Π_2^H describes the polarity/polarizability of solute i (Abraham *et al*, 1991), α_2^H describes the hydrogen-bonding acidity of solute i (Abraham *et al*, 1989) β_2^H describes the hydrogen bonding basicity of solute i (Abraham *et al*, 1990, 1993), and *V*x, describes the group-contributable molecular volume of solute i (Abraham *et al*, 1987) while *c*, *r*, *s*, *a*, *b*, and *v* are adjusted coefficients specific to the two-phase system, in this case air and water.

Equation 3.78 accurately predicted fuel-water partitioning of a wide range of nonpolar hydrocarbon and thiophene compounds in agreement with previous findings (Reckhorn *et al*, 2001). However, predictions were highly unreliable for polar solutes, since nonideal solvation typically occurs in cases where the solute and solution differ significantly in polarity and/or hydrogen-bonding capabilities (Prausnitz, 1969).

3.7 NUMERICAL METHODS

For most problems of practical importance it is not possible to find exact solutions by using analytical techniques. As a result, this has led to the development of numerical methods whereby the governing equation is transformed into a discrete form which then results in a series of algebraic equations which can be solved on a computer. The solution to the discrete problem represents an approximation to the solution of the governing equations. Various concepts have also been developed in an attempt to quantify how well the calculated numerical solution compares to the true solution. Many techniques are available for numerical simulation; they include the Runge-Kutta method, Newton-Raphson method, Finite Difference Methods (FDM), Finite Element Methods (FEM) and Finite Volume Methods (FVM) (Hirsch, 1998).

In order to quantify how well a particular numerical technique performs in solving a problem, there are four fundamental criteria that can be applied to compare and

contrast different methods. The four concepts are accuracy, consistency, stability and convergence. In theory these criteria apply to any form of numerical method though they are easily formulated for Finite Difference Methods (Lambert, 1991).

Accuracy is a measure of how well the discrete solutions represent the exact solution of the problems. Two quantities exist to measure this. The local or truncation error, which measures how well the different equation match the differential equations and the global error which reflects the overall error in the solution and in reality, is not possible to find unless the exact solution is known. An expression for the truncation error can be obtained by substituting the known exact solution of the problem into the discretization leaving a remainder which is then a measure of the error. Alternatively, the exact solution to the discretized problem could be substituted into the differential equation and the remainder obtained. For instance, for a partial differential equation (PDE), this would lead to an expression of the form $\tau = \mathrm{O}\left(\Delta t^q, \Delta x^p\right) = 0$ where τ is the truncation error and Δt and Δx are the time and spatial steps (assuming a regular grid). From this, the method is said to be q^{th} order in time and p^{th} order in space. Generally, this is referred to as the level of accuracy of the scheme (Hirsch, 1998). It is natural to assume that by decreasing the grid resolution, then any error will be reduced and this leads to the definition of consistency.

Mathematically, for a method to be consistent, the truncation error must decrease as the step size is reduced which is the case when q, p $\geq$ 1, which is equivalent to saying that as Δt , Δx tend to zero then the discretized equations should tend towards the differential equation. For a scheme to be of practical use, it must be consistent.

For a scheme to be stable, then any error will in the solution will remain bounded. In practice if an unstable method is used then the solution will tend towards infinity. Most methods have stability limits which place restrictions on the size of the grid spacing (i.e. Δt , Δx) that can be used. Physically, a stable method can be interpreted to be one where the grid points used in the calculation enclose the characteristic lines or domain of dependence. A number of methods are available for obtaining

expressions for the stability conditions and the appropriate choice depend on the actual problem (Smith, 1985).

Another requirement is that the numerical scheme should be convergent, which by definition means that the numerical solution should approach the exact solution as the grid spacing is reduced to zero. This is coupled with the global error. However, it is usually not possible to prove the convergence of a particular scheme to a specific problem. Instead use is made of Lax's Equivalence theorem which states that for a well posed initial value problem (IVP) and a consistent method, stability implies convergence in the case of a linear problem. For a non-linear equation, stability and consistency are necessary but not sufficient condition for convergence.

These criteria dictate whether a particular numerical scheme is suitable for solving a particular problem or not. There is another condition, which has to be satisfied in order to produce a valid solution and this related to the actual problem. The latter issue concerns how well define the problem is defined. In order to generate a numerical solution, the problem being considered must be well posed. For this to be the case then the following condition must hold

(a) a solution must exist
(b) the solution should be unique
(c) the solution should depend linearly on the data in some ways.

The last condition can be translated to mean that the solution should not be sensitive to small changes in the initial/boundary data of the problem. If a problem is well pose, then a valid numerical solution cannot be generated and any numerical treatment will fail or produce poor results. One easy way to produce an ill posed problem is to apply inappropriate boundary conditions, for example by trying to enforce values of the quantities being simulated on the characteristics leaving the computational domain. If the initial data is not fully specified, then this also presents an ill posed problem as there will be no unique solution (Formaggia *et al.,*, 2001).

3.7.1 Grid Generation and Explicit/implicit Formulation

An important factor in applying numerical techniques is the issue of Grid generation. Early efforts focused on using regular grids, whereby all the cells or elements were of the same size. This has advantages in terms of numbering the cells and forming discrete equations. For 2-d and 3-d problems, fitting a regular grid to complex geometries can often lead to problems. To overcome the difficulties created in implementing regular grids attention has moved towards irregular grids where the cell sizes may vary within the domain. Furthermore, unstructured (as opposed to structured) gridding has been introduced, leading to the ability to map any region. However, the resulting meshes generally have no apparent structure and so increase the level of complexity of generating suitable computer code. The predominant reason for using irregular gridding is the ability to concentrate the cells in areas where sharp gradients occur and so a high level of accuracy can be maintained throughout the region without the need to use a fine grid everywhere. If such a grid is generated at the onset of a problem, it may be that as the simulation progresses, the initial choice is no longer the most suitable and so this has led to the idea of adaptively whereby the grid evolves during simulation, in a manner determined by the numerical solution (Yee, 1987).

Another distinction that can be drawn between different methods is whether they are explicit or implicit. For instance, taking the linear advection equation.

$$u_t + au_x = 0 \qquad \text{a is a constant} \qquad 3.79$$

where u is a velocity component in the x direction. a is a scalar quantity.

If the solution is to be advanced to time level n+1, the spatial derivative may be approximate either in terms of the known value at time level n or the unknown quantities at level n+1. If an approximation for the spatial derivative is approximated at time level n, then that corresponds to an explicit method, whereas using level n+1 represents an implicit formulation. Explicit methods are generally simpler in terms of the resulting algebraic equations as implicit schemes usually require a matrix inversion which is more complex. However, most implicit schemes are not restricted by the stability constraints placed upon their explicit methods and so allow the use of much larger time steps (Kaw and Kalu, 2008).

3.7.2 Runge-Kutta Method

The Runge-Kutta method for solving differential equation is widely used and normally gives a high degree of accuracy. The Runge-Kutta method is as follows.

To solve $y' = f(x, y)$ 3.80

with initial condition y = y_0 at x = x_0, for a range of values of $x = x_0(h)x_n$.

Starting with x = x_0, y = y_0, y' = $(y')_0$ and h, we have

$$x_1 = x_0 + h \quad (3.81)$$

Finding y_1 requires four intermediate calculations

$$k_1 = hf(x_0, y_0) = h(y')_0 \quad (3.82)$$

$$k_2 = hf\left(x_0 + \frac{1}{2}h, y_0 + \frac{1}{2}k_1\right) \quad (3.83)$$

$$k_9 = hf\left(x_0 + \frac{1}{2}h, y_0 + \frac{1}{2}k_2\right) \quad (3.84)$$

$$k_4 = hf\left(x_0 + \frac{1}{2}h, y_0 + \frac{1}{2}k_3\right) \quad (3.85)$$

The increment Δy_0 in the y-value from x = x_0 to x = x_1 is then

$$\Delta y_0 = \frac{1}{6}(k_1 + 2k_2 + 2k_3 + k_4) \quad (3.86)$$

and finally $y_1 = y_0 + \Delta y_0$ 3.87

The method is very popular where accuracy of results is important. The main disadvantage of Runge-Kutta is that it does not readily lend itself to any self checking procedures (Arrowsmith *et al*, 1993).

3.7.3 Newton-Raphson Iterative Method

If x = x_0 is an approximate value of a root of the equation f(x) = 0, a better approximation x = x_1 is given by

$$x_1 = x_0 - \frac{f(x_0)}{f'(x_0)} \quad (3.88)$$

The process can be repeated and in general

$$x_{n+1} = x_n - \frac{f(x_n)}{f'(x_n)} \quad 3.89$$

There are times when the normal application of the Newton-Raphson method fails to converge to the required root. This is particularly so when f'(x_0) is very small. When this happens, the normal Newton-Raphson method fails and it is modified to give a second approximation x_1 further from the true root than x_0. In that case, x_1 can be obtained from the relationship

$$x_1 = x_0 \pm \sqrt{\frac{-2f(x_0)}{f''(x_0)}} \quad 3.90$$

we then revert to the normal relationship for subsequent iterations (Tjalling, 1995).

3.7.4 Finite Difference Method

Finite difference methods were the first techniques to be developed for approximating ordinary differential equations, and it is from such application that the theories regarding their properties have been generated.

Finite difference methods are based on performing Taylor series expansion and substituting the truncated expression into the differential equation. The idea is to approximate the differentials by differences in the solution at various points (Hirsch, 1998).

By definition,

$$u_x = \frac{\partial u}{\partial x} = \lim_{\Delta x \to 0} \frac{u(x+\Delta x) - u(x)}{\Delta x} \quad 3.91$$

when Δx is small this formula can be used as an approximation for the derivative of u at x. from Taylor's series

$$u(x+\Delta x) = u(x) + \Delta x u_x(x) + \frac{\Delta x^2}{2} u_{xx}(x) + \cdots \quad 3.92$$

and so by rearrangement

$$\frac{u(x+\Delta x) - u(x)}{\Delta x} = u(x) + \Delta x u_x(x) + \frac{\Delta x^2}{2} u_{xx}(x) + \cdots \quad 3.93$$

if Δx is small the successive terms in the expansion will decrease and so it is possible to write

$$u_x(x) = \frac{u(x+\Delta x) - u(x)}{\Delta x} + \mathrm{O}(\Delta x) \qquad 3.94$$

From the above equation, the leading term of the error in approximating u_x by the right hand side is of order Δx and so this represents a first order approximation. It is possible to define order difference formula to approximate derivatives and these may have different order of accuracy. The above analysis deals with the continuous solution, however, the objective is to calculate u at a set of discrete point on the mesh, and this is the numerical solution. Let the mesh points be denoted by x_i where $i = 1,2,3\cdots N$ and the region have been discretized into N equally sized elements of length Δx. Then the numerical u_i can be thought of as point value where $u_i = u(i\Delta x)$. Following this notation, there are three common ways to approximate the first derivative of u with respect to x.

(i) Forward difference

$$(u_x)_i = \frac{u_{i+1} - u_i}{\Delta x} + \mathrm{O}(\Delta x) \qquad 3.95$$

(ii) Backward difference

$$(u_x)_i = \frac{u_i - u_{i-1}}{\Delta x} + \mathrm{O}(\Delta x) \qquad 3.96$$

(iii) Central difference

$$(u_x)_i = \frac{u_{i+1} - u_{i-1}}{2\Delta x} + \mathrm{O}(\Delta x^2) \qquad 3.97$$

as can be seen, both forward and backward differences are first order approximations whereas the central difference is second order as can be shown by Taylor series analysis. These formulae have different merits and the best choice depends on the problem being simulated. In the case of ordinary differential equations, many other difference formulae can be derived and standard techniques are available for doing so. However, for partial differential equations, most schemes are based on using standard forward, backward and central differences.

In this study, the implicit finite Difference Method was used as a matter of convenience and because it is accurate, consistent and stable. Equations arising

from implicit method are difficult to solve, but their solution is not restricted by stability criteria. There is also no restriction on the size of the time step, Δt.

3.7.5 Algorithm for Solving the Kinetic Model without Mass Transfer

i. Discretize the equation using Implicit Finite Difference Method
ii. Read in the data; k, C_0, K_M, t
iii. Compute the concentration of substrate at different time

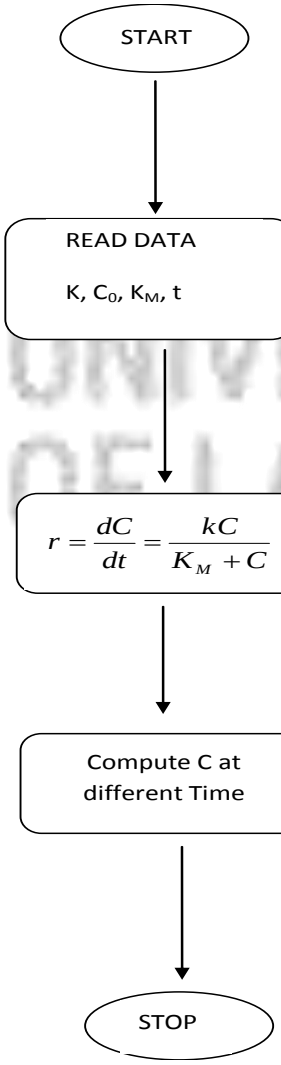


Figure 3.2: Flow Chart of the Method of Computing Substrate Concentration at Different Times from the Kinetic Model without Mass Transfer Using the Finite Difference Method

3.7.6 Algorithm for Solving the Mass Transfer Influenced Kinetic Model

i. Discretize the equation using Implicit Finite Difference Method

ii. Read in the data; k, C_0, K_M, t, K_D

iii. Compute the concentration of substrate at different time

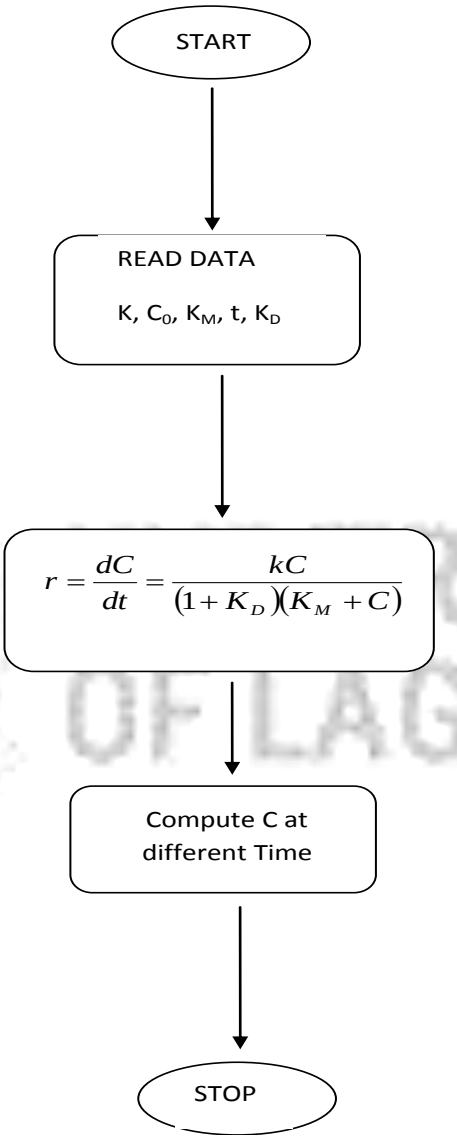


Figure 3.3: Flow Chart of the Method of Computing Substrate Concentration at Different Times from the Mass Transfer Influenced Kinetic Model Using the Finite Difference Method

The sub-routines of the flow charts can be found in Appendix F

CHAPTER 4

4.0 RESULTS

The results of the biodesulfurization of diesel and kerosene by the isolated microorganisms are presented in this chapter. The results of the series of biochemical and morphological tests carried out on the isolated microorganisms as shown in Table 4.1 are also presented. Furthermore, the comparison of the predicted substrate conversion – time profiles with the experimental data are also presented in this chapter

4.1 IDENTIFICATION OF MICROORGANISMS

Series of biochemical and morphological tests were carried out on the isolated microorganisms in order to identify them. Two isolates were selected for studies having shown good growth in SRB medium. The two isolates were designated A and D for easy identification and their population densities in the soil samples were 6.40 x 10^6 cfu/g and 6.32 x 10^6 cfu/g respectively. The two isolates were found to have a shape varying from oval to rod with smooth edges and a raised elevation on nutrient agar with cream color. They were also observed to be Gram-negative due to their inability to retain the purple color of the basic strain and, therefore, appeared pink under the light microscope. Their growth on sodium lactate was very poor but responded positively to the hydrogen sulfide test. This was ascertained by the black coloration of the lead acetate paper used for the test. Isolate A was able to produce indole from the utilization of tryptophan; on the other hand, isolate D was not able to do same. The two isolates tested negative to the citrate and urease tests. They also tested negative to the catalase test, an indication that the isolates lack the ability to produce catalase capable of catalyzing the breakdown of hydrogen peroxide to form oxygen and water. Further, the isolates tested negatively to the Methyl Red-Voges Proskauer tests. The two isolates tested positively to some sugar fermentation tests and negatively to other sugars such as maltose and xylose. The two isolates utilized glucose, mannitol and maltose. Isolate A could utilize lactose while D could not.

Table 4.1: The Response of the Isolates to the various Biochemical Tests

Cell Morphology and Results Of Biochemical Tests.	Isolate A	Isolate D
Colonial Characteristics on SRB Medium.	Smooth edge, raised Elevation & cream in color	Smooth edge, raised Elevation & cream in color
Shape of cells	Oval to rod-shaped	Oval to rod shaped
Gram Stain	-	-
Sodium lactate utilization	VP	VP
H_2S Production	+	+
Indole (Tryptophan utilization)	+	-
Urease Test	-	-
Citrate Test	-	-
Catalase test	-	-
MRVP(Methyl Red)	-	-
MRVP(Voges- Proskauer)	-	-
Motility	+	-
Glucose	+	+
Xylose	-	+
Mannitol	+	+
Maltose	+	+
Lactose	+	-
Gas Production From Glucose	+	+
Sodium propionate	+	+
Sodium anilinate	-	+
Sodium benzoate	-	+
Sodium butyrate	-	+
Presumptive Identification of Isolates.	*Desulfobacterium indolicum*	*Desulfobacterium anilini*

+, Positive reaction/growth; -, Negative reaction/no growth; VP, Very poor growth

On the other hand, isolate D utilized xylose while A did not. The two isolates were differentiated based on the latter observations. These isolates tested negative to the Methyl Red Voges Proskauer (MRVP) tests.

This implies that the isolates are inactive at a pH of around 4.5 and thus need to be sustained at an optimum pH of between 6.5 and 7. Most sulfur-utilizing bacteria are known to exhibit this trait. The isolates, A and D tested positively to the hydrogen sulfide test and negatively to the urease test meaning that they do not contain the enzyme urease which is responsible for splitting urea to ammonia.

Thus, isolate A was tentatively identified as *Desulfobacterium indolicum* while isolate D as *Desulfobacterium anilini* based on their differential morphological and biochemical characteristics (Table 4.1) and by reference to *Bergey's Manual of Determinative Bacteriology* (Holt *et al*., 1994). They belong to the same family of sulfur-utilizing bacteria but of different species based on this difference in their responses to biochemical tests particularly the utilization of sodium anilinate and indole production from tryptophan.

4.4 KINETIC PARAMETER ESTIMATION

The kinetic parameters for the biodesulfurization of diesel and kerosene were estimated by subjecting the experimental data to the procedures described in sub-section 3.6. The experimental data were subjected to all the linear kinetic equations namely; the Hanes, Lineweaver-Buck and Eadie-Hofstee Plots. The values of k and K_M obtained are shown in the Tables 4.2. The kinetic parameters used in the simulations used in this study were obtained by subjecting the ones obtained from the linear plots to non-linear regression analysis, the obtained parameters are shown in Table 4.3. The distribution coefficient was estimated through the efficacy of combining linear solvation energy relationships (LSERs) developed for pure systems via application of linear solvent strength theory (Arey and Gschwend, 2005). The detailed procedure for the estimation is clearly shown in appendix E

Table 4.2: Estimated Kinetic Parameters for the Biodesulfurization of Diesel and Kerosene

	Hanes Plot	Lineweaver-Burk	Eadie-Hofstee Plot
Maximum Rate Constant, $k_{BT,}$ for *D indolicum,* (mg/L.hr)	0.554	0.550	0.553
Michaelis-Menten constant, $K_{M, BT,}$ for *D indolicum* Diesel (mg/L)	19.020	18.890	18.990
Maximum Rate Constant, $k_{DBT,}$ for *D indolicum*, (mg/L.hr)	5.990	5.992	5.992
Michaelis-Menten constant, $K_{M, DBT,}$ for *D indolicum* Diesel (mg/L)	192.6	192.8	192.6
Maximum Rate Constant, $k_{BT,}$ for *D anilini,* (mg/L.hr)	0.554	0.547	0.554
Michaelis-Menten constant, $K_{M, BT,}$ for *D anilini* Diesel (mg/L)	18.650	18.380	18.620
Maximum Rate Constant, $k_{DBT,}$ for *D anilini*, (mg/L.hr)	6.116	6.110	6.114
Michaelis-Menten constant, $K_{M, DBT,}$ for *D anilini* Diesel (mg/L)	182.1	181.9	182.1
Maximum Rate Constant, $k_{TH,}$ for *D indolicum,* (mg/L.hr)	1.03	1.03	1.03
Michaelis-Menten constant, $K_{M, TH,}$ for *D indolicum* Kerosene (mg/L)	0.574	0.560	0.561
Maximum Rate Constant, $k_{DMT,}$ for *D indolicum*, (mg/L.hr)	1.350	1.348	1.349
Michaelis-Menten constant, $K_{M, DMT,}$ for *D indolicum* Kerosene (mg/L)	54.68	54.52	54.66
Maximum Rate Constant, $k_{TH,}$ for *D anilini,* (mg/L.hr)	0.104	0.104	0.104
Michaelis-Menten constant, $K_{M, TH,}$ for *D anilini* Kerosene (mg/L)	0.556	0.553	0.554
Maximum Rate Constant, $k_{DMT,}$ for *D anilini*, (mg/L.hr)	1.426	1.426	1.426
Michaelis-Menten constant, $K_{M, DMT,}$ for *D anilini* Kerosene (mg/L)	51.76	51.78	51.78

Table 4.3: Kinetic and Mass Transfer Parameters used for the modeling of Biodesulfurization of Diesel and Kerosene

	k (mg/L.hr)	K_M (mg/L)	K_D
Thiophene by *D anilini*	0.104	0.548	1.281
Thiophene by *D indolicum*	0.103	0.575	1.281
2,5 Dimethylthiophene by *D anilini*	1.426	51.700	1.054
2,5 Dimethylthiophene by *D indolicum*	1.350	54.700	1.054
Benzothiophene by *D anilini*	0.572	18.050	2.312
Benzothiophene by *D indolicum*	0.540	19.837	2.312
Dibenzothiophene by *D anilini*	6.118	182.278	2.038
Dibenzothiophene by *D indolicum*	5.992	192.782	2.038

4.5 RESULT OF BIODESULFURIZATION REACTIONS

This section presents the experimental and model simulation results of the biodesulfurization of diesel and kerosene in the presence of isolated bacteria, *Desulfobacterium indolicum* and D*esulfobacterium anilini.*

The results of the experimental investigation of the biodesulfurization of diesel and kerosene in the presence of *Desulfobacterium anilini* and D*esulfobacterium indolicum* are presented graphically in Figures 4.1 to 4.24. Figures 4.1 to 4.8 show the substrate concentration versus time data obtained for the biodesulfurization of diesel and kerosene. The sulfur-containing organic components were sorted by their respective retention times and their amount by appropriate calibration of their concentration with the area under the peak at various times of the chromatograms shown in appendix A. The retention times for Thiophene, 2, 5 – Dimethylthiophene, Benzothiophene and Dibenzothiophene are 14.935, 16.428, 21.608 and 24.941 minutes respectively. The concentration of the organosulfur compounds were obtained by calculating the area under the peak with the use of enhanced integrator. The obtained values are shown in Tables B.1 to B.4 in appendix B. The obtained

values were subjected to the student's t-test statistical analysis. They were found to be significant at the confidence of 0.95 the detailed results of the analysis can be found in Tables C.1 to C.8 in Appendix C. Figures 4.9 to 4.16 show the results of the simulation of the kinetic model for the biodesulfurization of diesel and kerosene without influence of mass transfer. The simulated substrate concentration versus time data obtained for the mass transfer-controlled kinetic model for the biodesulfurization of diesel and kerosene are shown in Figures 4.17 to 4.24.

Figure 4.1 shows the conversion versus time data for the biodesulfurization of thiophene in kerosene by *Desulfobacterium anilini*. The latter plot showed that the substrate was desulfurized steadily from its initial concentration of 6.955 mg/L to 0.998 mg/L in 72 hours of desulfurization. This translates to over 85% desulfurization. Kinetic analysis of this experimental data resulted in a maximum rate constant k of 0.052 mg/L.hr and a Michaelis-Menten constant K_M of 0.548 mg/L for the biodesulfurization of thiophene in kerosene by *Desulfobacterium anilini*.

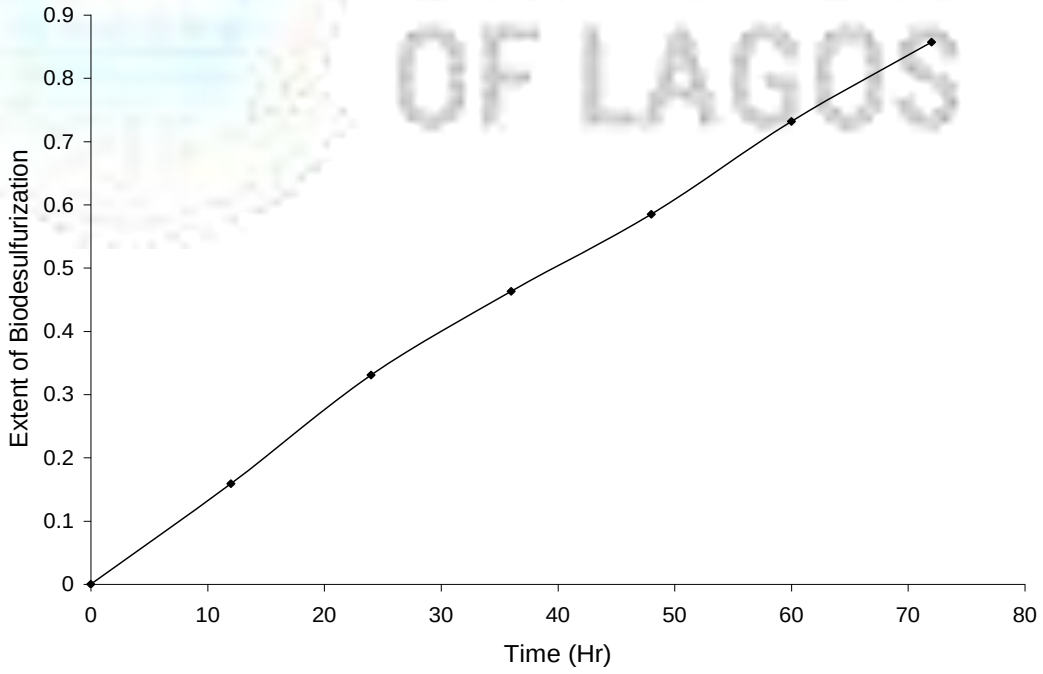


Figure 4.1: Biodesulfurization of Thiophene in Kerosene by *D. anilini*

The biodesulfurization of 2, 5-Dimethylthiophene in kerosene by D *anilini* is shown in Figure 4.2. It showed that 2, 5-Dimethylthiophene was also steadily desulfurized by

the organism. However, only 73% of the substrate was desulfurized. The concentration of the 2, 5-Dimethylthiophene was reduced from 41.724 mg/L to 11.376 mg/L after 72 hours. Kinetic analysis of the experimental data revealed a maximum rate constant k of 0.713 mg/L.hr and a Michaelis-Menten constant K_M of 51.700 mg/L for the biodesulfurization of 2, 5-Dimethylthiophene in kerosene by D *anilini*.

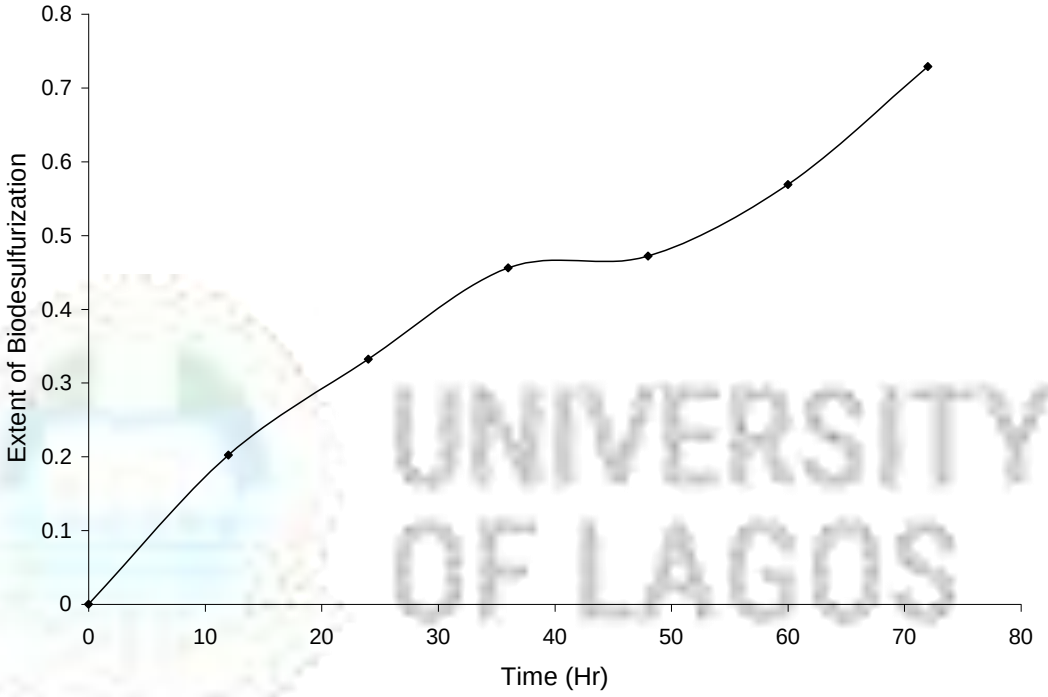


Figure 4.2: Biodesulfurization of 2, 5 Dimethylthiophene in Kerosene by *D. anilini*

Interestingly, *Desulfobacterium indolicum* also desulfurized the sulfur-containing organic compounds in kerosene. The extent of biodesulfurization-time profiles are shown in Figures 4.3 and 4.4.

Figure 4.3 showed the biodesulfurization of thiophene in kerosene by *Desulfobacterium indolicum*. It also showed that the thiophene was desulfurized steadily. Its initial concentration in the kerosene was 6.955 mg/L but was reduced to 1.105 mg/L after 72 hours of desulfurization. This translates to over 84% desulfurization. Kinetic analysis on this experimental data revealed a maximum rate constant k of 0.0515 mg/L.hr and a Michaelis-Menten constant K_M of 0.575 mg/L.

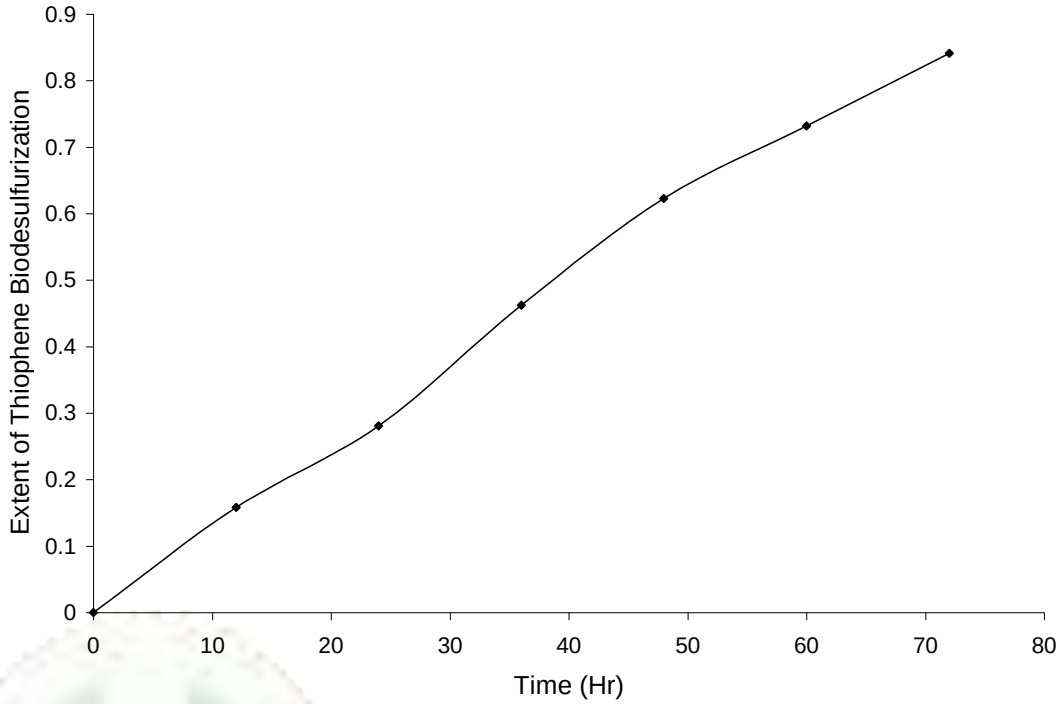


Figure 4.3: Biodesulfurization of Thiophene in Kerosene by *D. indolicum*

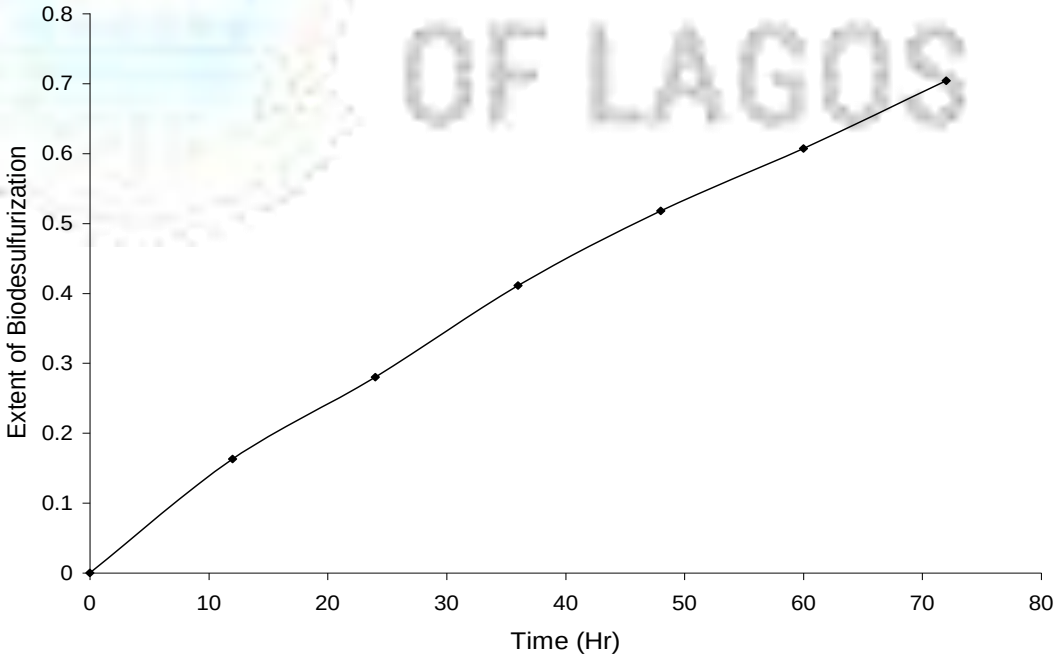


Figure 4.4: Biodesulfurization of 2, 5 Dimethylthiophene in Kerosene by *D. Indolicum*

Figure 4.4 showed the biodesulfurization profile of 2, 5 Dimethylthiophene in Kerosene by D *indolicum.* The Figure showed that the substrate concentration was

steadily reduced from 41.724 to 12.656 mg/L in 72 hours. This is about 70% biodesulfurization of the 2, 5 Dimethylthiophene in Kerosene. The kinetic analysis on the profile resulted in its maximum rate constant, k to be 0.675 mg/L.hr and the Michaelis-Menten constant, K_M of 54.700 mg/L. On the overall, 72% of kerosene was desulfurized by *Desulfobacterium indolicum*.

Generally, *Desulfobacterium anilini* was found to desulfurize kerosene better than *Desulfobacterium indolicum*.

Interestingly, the cells of *Desulfobacterium indolicum* and *Desulfobacterium anilini* were also found capable of significantly desulfurizing diesel. The profiles of biodesulfurization conversion with time of the sulfur heterocycles are shown in Figures 4.5 to 4.8.

The biodesulfurization of Dibenzothiophene (DBT) by *D anilini* is shown in Figure 4.5.

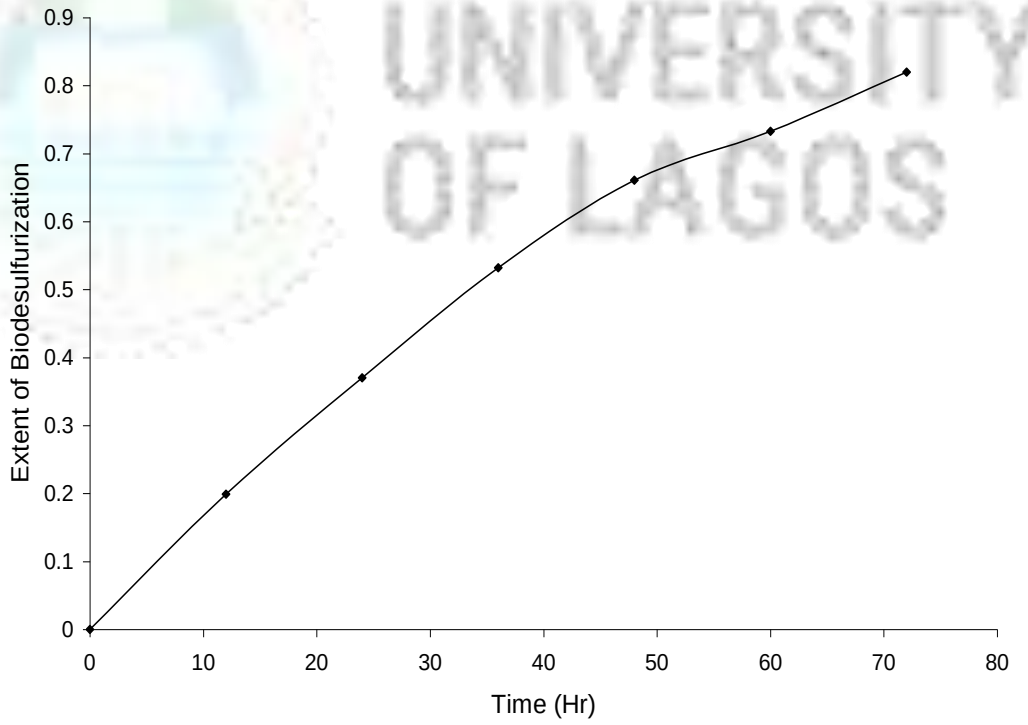


Figure 4.5: Biodesulfurization of Dibenzothiophene in Diesel by *D. anilini*

The concentration of the DBT was reduced to 28.318 mg/L from an initial concentration of 157.031 mg/L in 72 hours. This represents 82% biodesulfurization of DBT in diesel. Further, kinetic parameters of the reaction were estimated as 3.059

mg/L.hr for the maximum rate constant, k and 182.27 mg/L for the Michaelis-Menten constant, $K_{M.}$

Further, 81% of benzothiophene was desulfurized due to the action of *D anilini* on diesel. Its concentration was reduced to 1.681 mg/L from an initial concentration of 9.006 mg/L. The profile of the biodesulfurization is shown in Figure 4.6. The maximum rate attainable for the reaction is 0.270 mg/L.hr and the Michaelis-Menten constant is 18.050 mg/L.

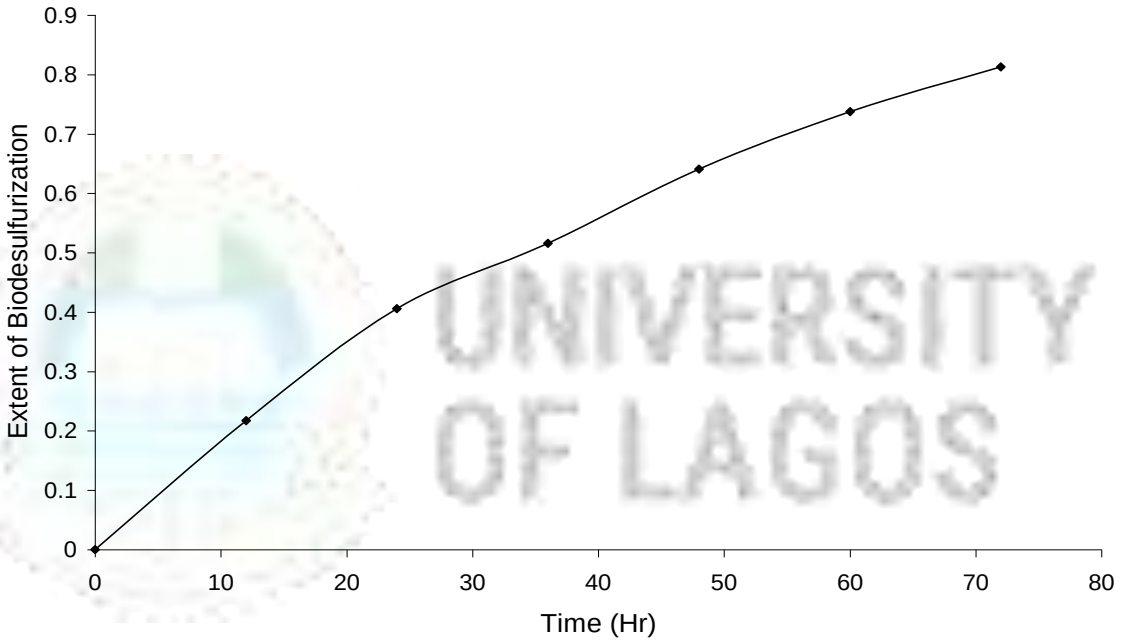


Figure 4.6: Biodesulfurization of Benzothiophene in Diesel by *D. anilini*

Similarly, *Desulfobacterium indolicum* was also found capable of desulfurizing diesel. The profiles for the biodesulfurization of Dibenzothiophene and benzothiophene by the microorganism are shown in Figures 4.7 and 4.8 respectively. The concentration of the DBT was reduced to 31.692 mg/L from an initial concentration of 157.031 mg/L in 72 hours, representing about 80% biodesulfurization of DBT in diesel (Figures 4.7).

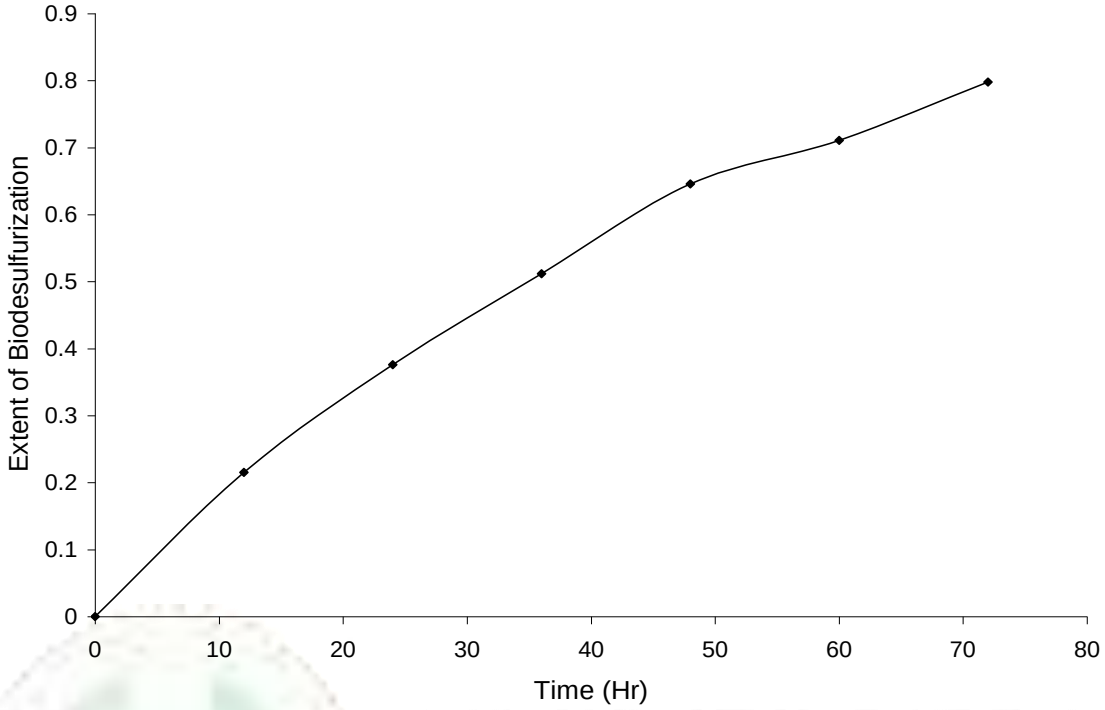


Figure 4.7: Biodesulfurization of Dibenzothiophene in Diesel by *D. indolicum*

Furthermore, kinetic parameters of the reaction were estimated at 2.996 mg/L.hr for the maximum rate constant, k. The estimated Michaelis-Menten constant, $K_{M.}$ was 192.78 mg/L. The microorganism succeeded in desulfurizing about 82% of benzothiophene in diesel in 72 hours. It reduced its concentration to 1.72 mg/L from an initial of 9.006 mg/L. The profile is shown in Figure 4.8. The maximum rate constant obtainable in this reaction was found to be 0.286 mg/L.hr and its Michaelis-Menten constant to be 19.837 mg/L.

Generally, *Desulfobacterium indolicum* desulfurized diesel very well by reducing its sulfur content from 166.037 mg/L to 33.412 mg/L representing 80% of the sulfur content analyzed. Comparative studies of the biodesulfurizing capability of the microorganisms revealed that the *Desulfobacterium anilini* performed better than *Desulfobacterium indolicum* even though the two microorganisms did well.

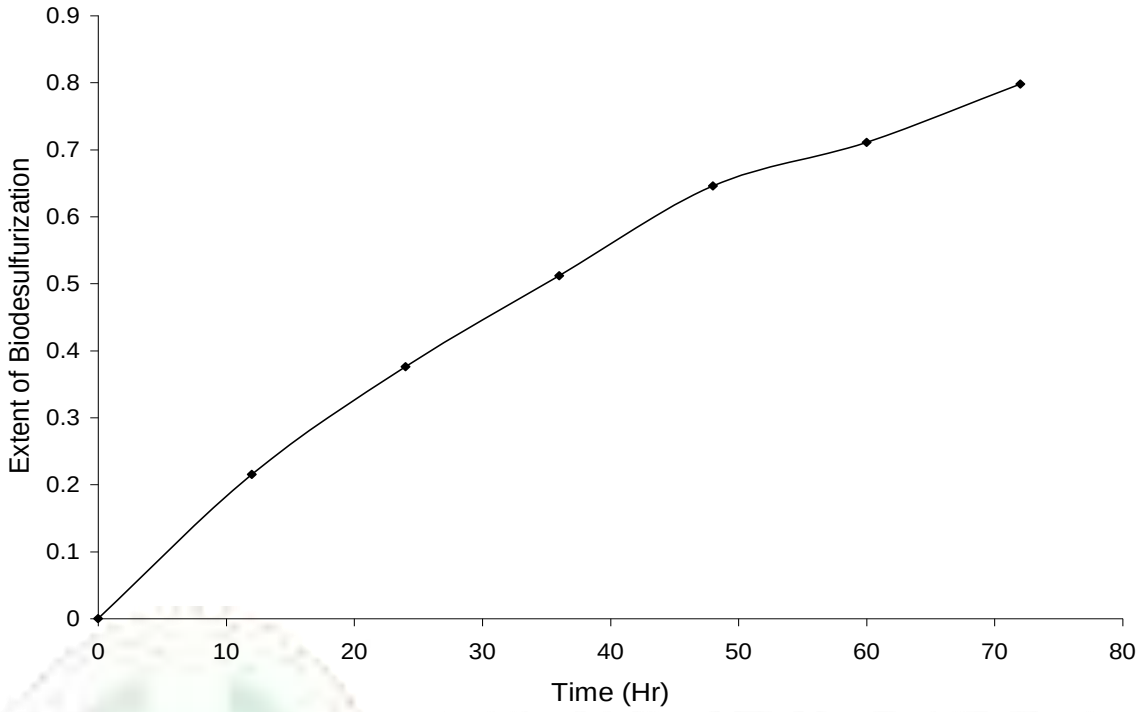


Figure 4.8: Biodesulfurization of Benzothiophene in Diesel by *D. indolicum*

Figures 4.9 to 4.16 showed the comparison of experimental and predicted extent of biodesulfurization – time profiles based on kinetic model without mass transfer with the experimental data. The kinetic parameters used in the simulation of the models were obtained by subjecting the experimental data to non-linear regression analysis. The distribution coefficient on the other hand was estimated by combining linear solvation energy relationships (LSERs) developed for pure systems via application of linear solvent strength theory (Arey and Gschwend, 2005).The level of agreement between the simulated and experimental data was determined by the sum of variances between the sets of data. The sum that is lower between two sets of data has a better agreement.

Figures 4.9 and 4.10 showed the comparison of experimental and simulated conversion – time profiles during the biodesulfurization of kerosene by *Desulfobacterium anilini* kinetic model without mass transfer.

Figure 4.9 showed the thiophene conversion – time profile in kerosene biodesulfurization. The deviations between the experimental and simulated data at the sample points range from 0.027 and 0.073. The overall sum of the variances at these points is 1.686 x 10^{-2}.

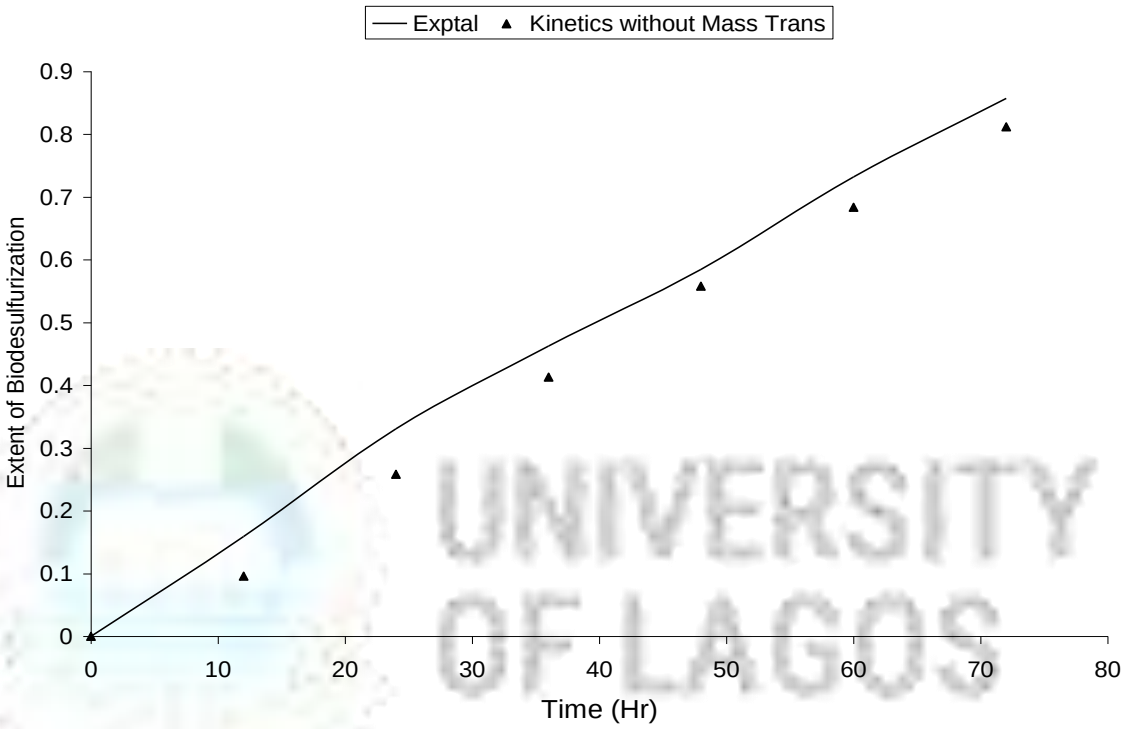


Figure 4.9: The Experimental and Simulated Conversion of Thiophene -Time Profile during Kerosene Biodesulfurization by *D. anilini* (Kinetic Model without Mass Transfer).

For the 2, 5 – Dimethylthiophene conversion - time profile in kerosene biodesulfurization, the deviations ranged between 0.001 and 0.088 and the overall sum of the variances at these points is 2.204 x 10^{-2}. The profile is shown in Figure 4.10.

It is clear from Figures 4.9 and 4.10 that the conversion – time profile for thiophene showed better agreement than for 2, 5 – Dimethylthiophene because the sum of the variances for the thiophene profile is lower than that for 2, 5 – Dimethylthiophene.

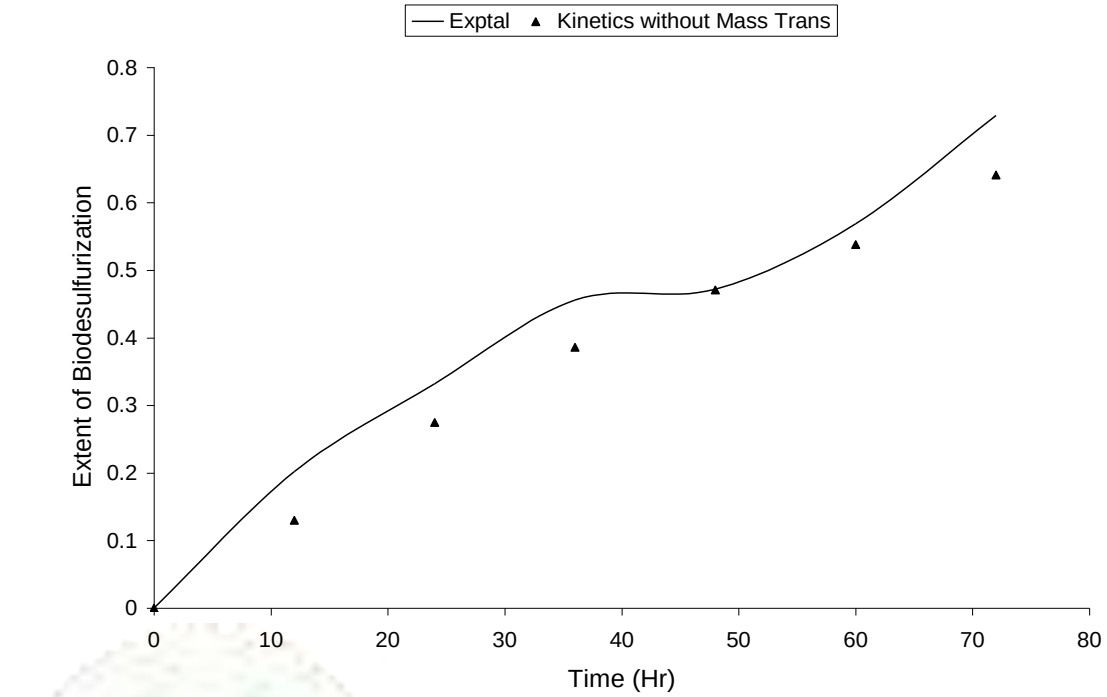


Figure 4.10: The Experimental and Simulated Conversion of 2, 5 - Dimethylthiophene -Time Profile during Kerosene Biodesulfurization by *D. anilini* (Kinetic Model without Mass Transfer).

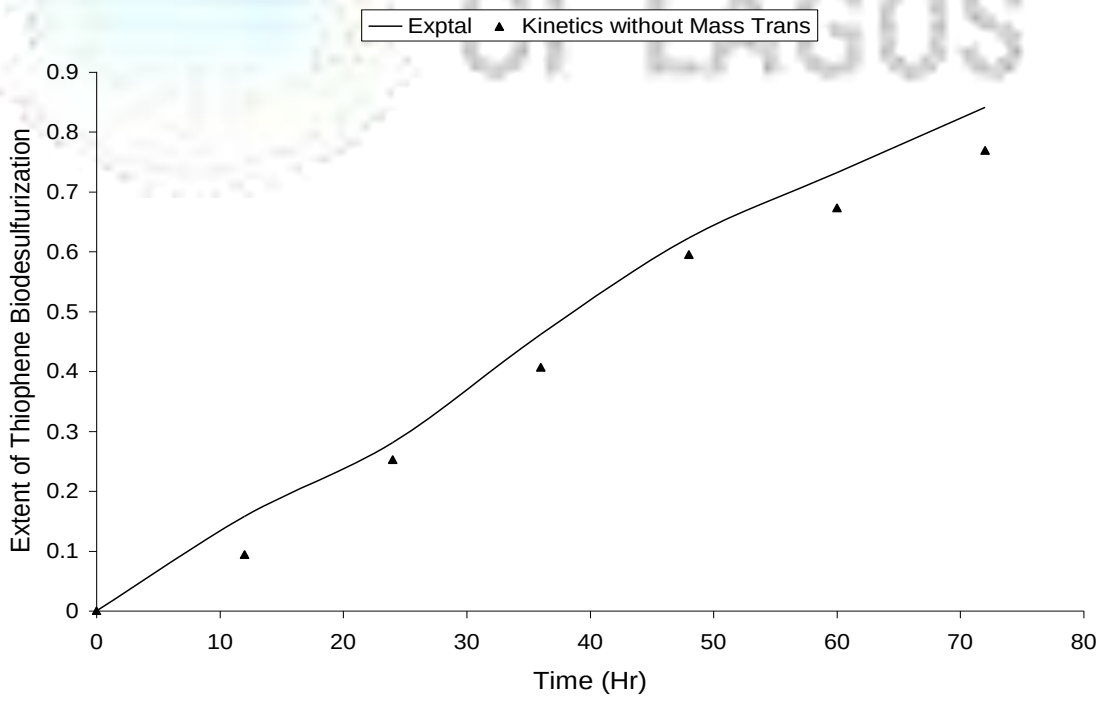


Figure 4.11: The Experimental and Simulated Conversion of Thiophene -Time Profile during Kerosene Biodesulfurization by *D. indolicum* (Kinetic Model without Mass Transfer).

Similarly, Figure 4.11 showed the comparison of experimental and simulated conversion – time profiles during the biodesulfurization of kerosene by *Desulfobacterium indolicum* using kinetic model without mass transfer.

The deviations between the experimental and simulated data at the sample points range from 0.028 with 0.072. The overall sum of the variances at these points is 1.735×10^{-2}.

Figure 4.12 shows the simulated 2, 5 – Dimethylthiophene biodesulfurization profile in Kerosene by *Desulfobacterium indolicum.* The deviations range from 0.030 to 0.106. The overall sum of the variances at these points is $2.569 \times 10^{-2.}$.

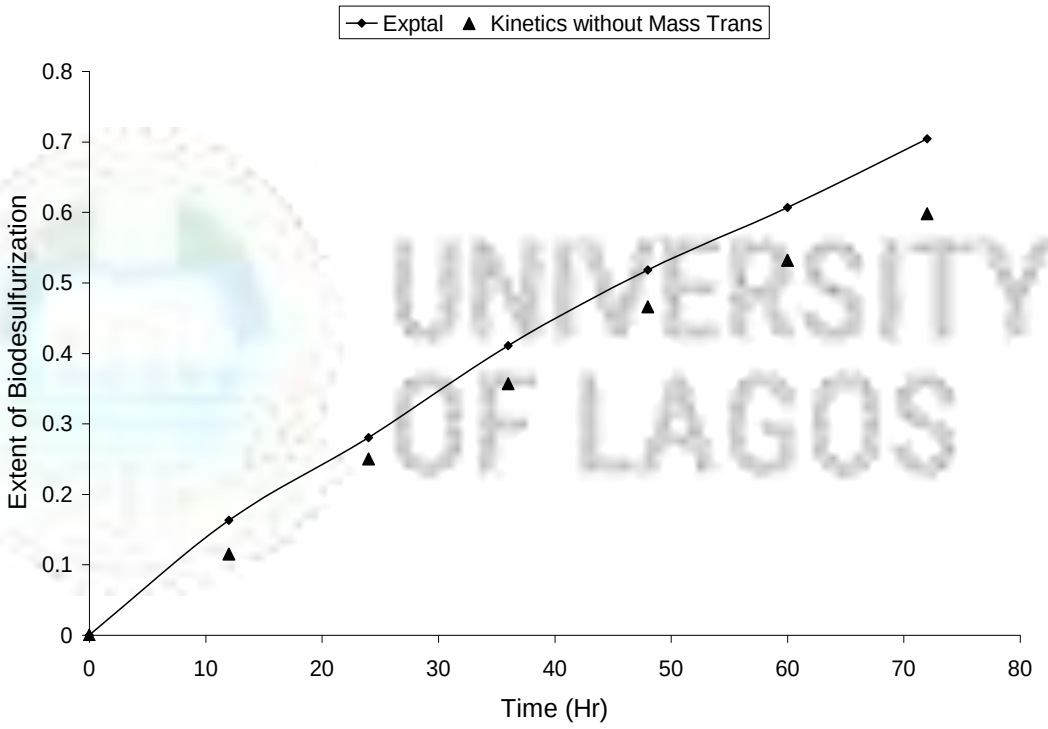


Figure 4.12: The Experimental and Simulated Conversion of 2, 5 - Dimethylthiophene -Time Profile during Kerosene Biodesulfurization by *D. indolicum* (Kinetic Model without Mass Transfer).

It is also obvious from Figures 4.11 and 4.12 that the conversion – time profile for thiophene during the biodesulfurization of kerosene by *Desulfobacterium indolicum* showed better agreement than for 2, 5 – Dimethylthiophene because the sum of the variances for the thiophene profile than that for 2, 5 – Dimethylthiophene.

Generally for the simulation of kerosene biodesulfurization by *Desulfobacterium anilini* (Figures 4.9 and 4.10) and *Desulfobacterium indolicum* (Figures 4.11 and 4.12), the sum of variances range from 1.686 x 10^{-2} to 2.569 x 10^{-2} . The deviations from points also range from 0.001 to 0.106. One may then infer that the simulated data for thiophene biodesulfurization by *Desulfobacterium anilini* is best while the best data point obtained from the kinetic model is 0.001 from the biodesulfurization of 2, 5 – Dimethylthiophene by *Desulfobacterium anilini*.

Similarly, Figures 4.13 and 4.14 showed the comparison of experimental and simulated conversion – time profiles during the biodesulfurization of diesel by *Desulfobacterium anilini* using kinetic model without mass transfer.

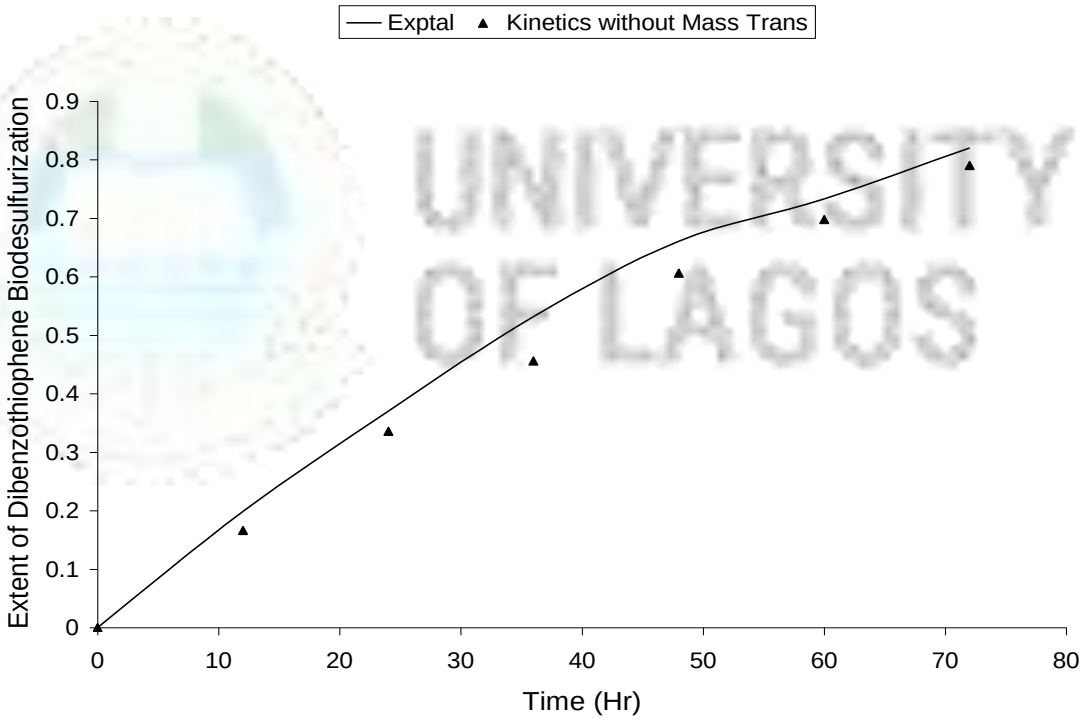


Figure 4.13: The Experimental and Simulated Conversion of Dibenzothiophene -Time Profile during Diesel Biodesulfurization by *D. anilini* (Kinetic Model without Mass Transfer).

The sum of variances for Dibenzothiophene conversion – time profile during biodesulfurization of diesel is 1.317 x 10^{-2}. The deviations between the experimental

and simulated data at the sample points range from 0.030 to 0.076. The profile was shown in Figure 4.13.

Figure 4.14 showed the benzothiophene conversion – time profile during biodesulfurization of diesel. The comparison of the simulated data with the experimental data gave a sum of variances of 6.566 x 10^{-3} with the data points ranging from 0.009 to 0.047.

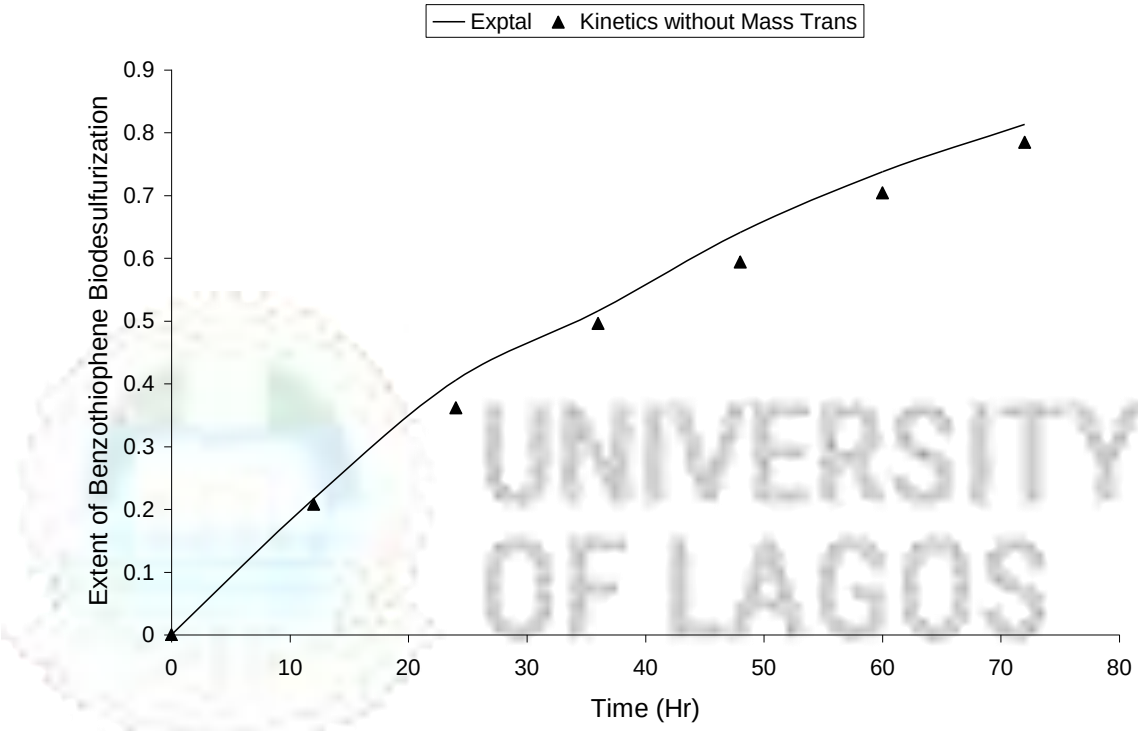


Figure 4.14: The Experimental and Simulated Conversion of Benzothiophene -Time Profile during Diesel Biodesulfurization by *D. anilini* (Kinetic Model without Mass Transfer).

It is obvious from Figures 4.13 and 4.14 that the conversion – time profile for benzothiophene showed better agreement with the experimental profile than for Dibenzothiophene because the sum of the variances of the former profile is lower than that of the latter.

Figures 4.15 and 4.16 also showed the comparison of experimental and simulated conversion – time profiles during the biodesulfurization of diesel by *Desulfobacterium indolicum* using kinetic model without mass transfer.

Figure 4.15 showed the Dibenzothiophene conversion – time profile in diesel biodesulfurization. The deviations between the experimental and simulated data at the sample points range from 0.007 and 0.052. The overall sum of the variances at these points is 4.539 x 10^{-3}.

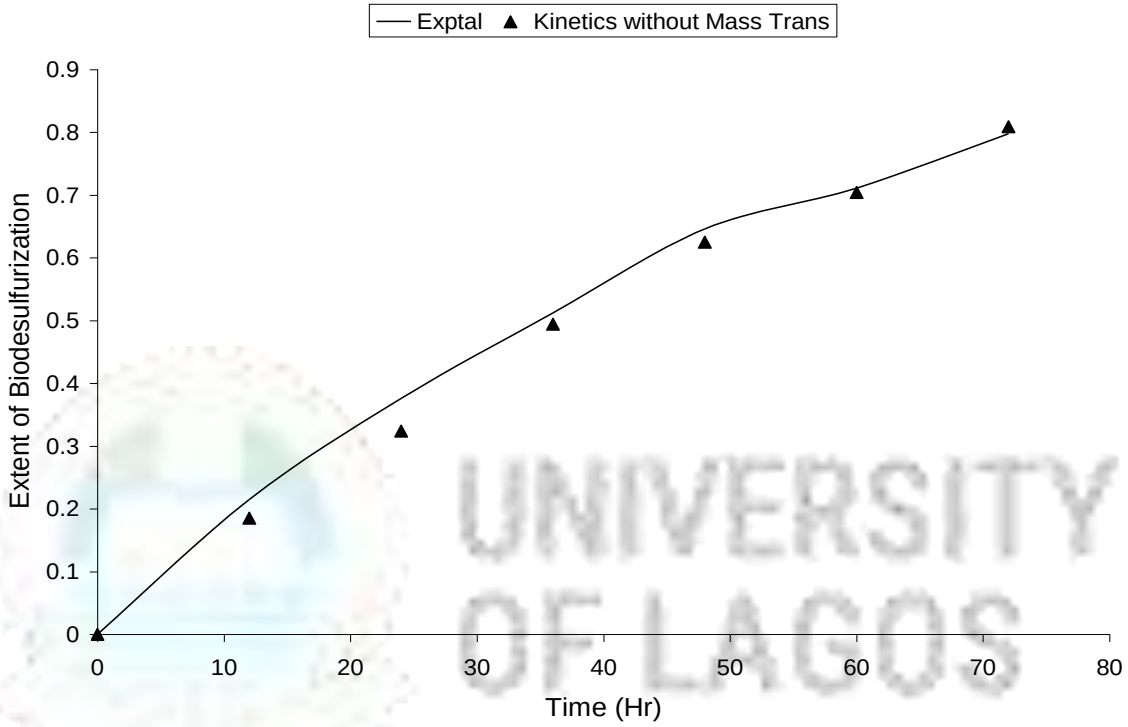


Figure 4.15: The Experimental and Simulated Conversion of Dibenzothiophene -Time Profile during Diesel Biodesulfurization by *D. indolicum* (Kinetic Model without Mass Transfer).

Figure 4.16 showed the simulated benzothiophene conversion - time profile in the biodesulfurization of diesel by *Desulfobacterium indolicum.* The deviations range from 0.017 to 0.084. The overall sum of the variances at these points is 1.849 x $10^{-2.}$.
It is clear from Figures 4.15 and 4.16 that the conversion – time profile for dibenzothiophene showed better agreement with the experimental profile than for benzothiophene because the sum of the variances of the dibenzothiophene profile is lower than that of the benzothiophene. This observed trend is different from that observed with same substrates by *Desulfobacterium anilini*.
Generally for the simulation of biodesulfurization of diesel by *Desulfobacterium anilini* (Figures 4.13 and 4.14) and *Desulfobacterium indolicum* (Figures 4.15 and 4.16), the

sum of variances range from 4.539 x 10^{-3} to 1.849 x 10^{-2} . The deviations from points also range from 0.007 to 0.084. One may then conclude that the simulated data for dibenzothiophene biodesulfurization by *Desulfobacterium indolicum* is best while the best data point obtained from the kinetic model is 0.007 from the biodesulfurization of dibenzothiophene by *Desulfobacterium indolicum*.

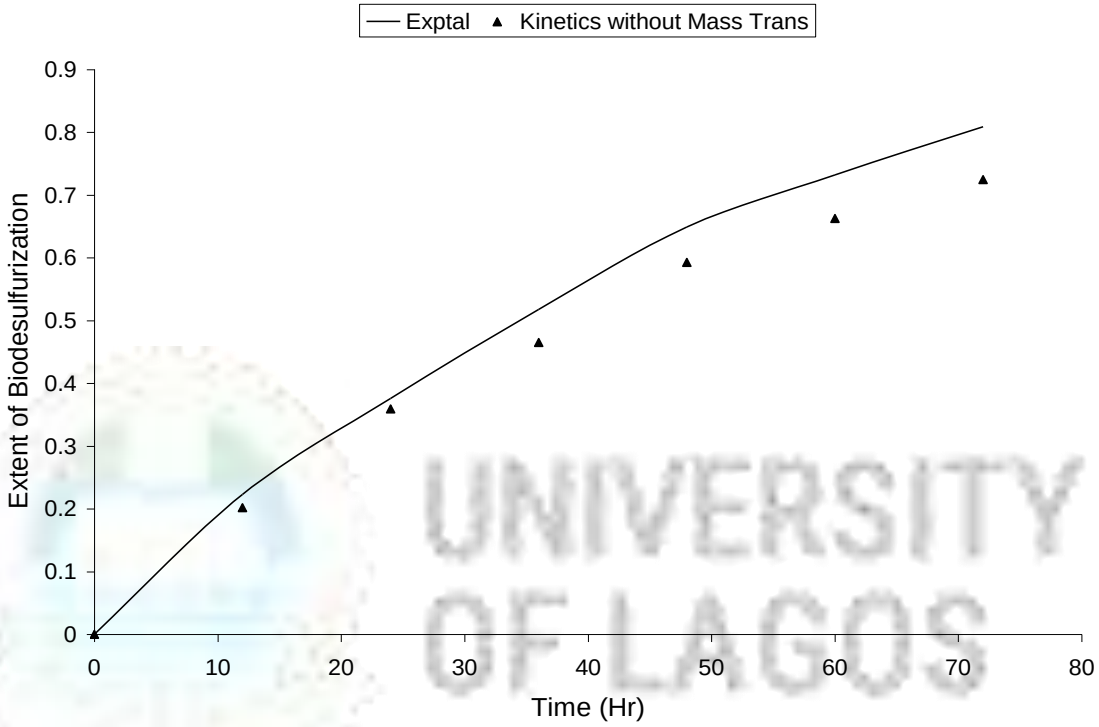


Figure 4.16: The Experimental and Simulated Conversion of Benzothiophene -Time Profile during Diesel Biodesulfurization by *D. indolicum* (Kinetic Model without Mass Transfer).

There are three profiles in each of the Figures labeled 4.17 to 4.24. They showed the comparison of experimental and predicted extent of biodesulfurization – time profiles based on kinetic model without mass transfer on one hand, and a mass transfer influenced kinetic model on the other hand with the experimental data.

Figures 4.17 and 4.18 showed the comparison of experimental and simulated conversion – time profiles during the biodesulfurization of kerosene by *Desulfobacterium anilini* using kinetic model without mass transfer and mass transfer influenced kinetic model.

Figure 4.17 showed the thiophene conversion – time profile in kerosene biodesulfurization. The deviations between the experimental and simulated data at the sample points range from 0.027 and 0.073 for kinetic model without mass transfer. The overall sum of the variances at these points is 1.686 x 10^{-2}. For the mass transfer influenced kinetic model the sum of variances of the simulated points compared to that of the experimental is 2.587 x 10^{-3} while the deviation between the experimental and the simulated data points ranged from 0.001 to 0.033.

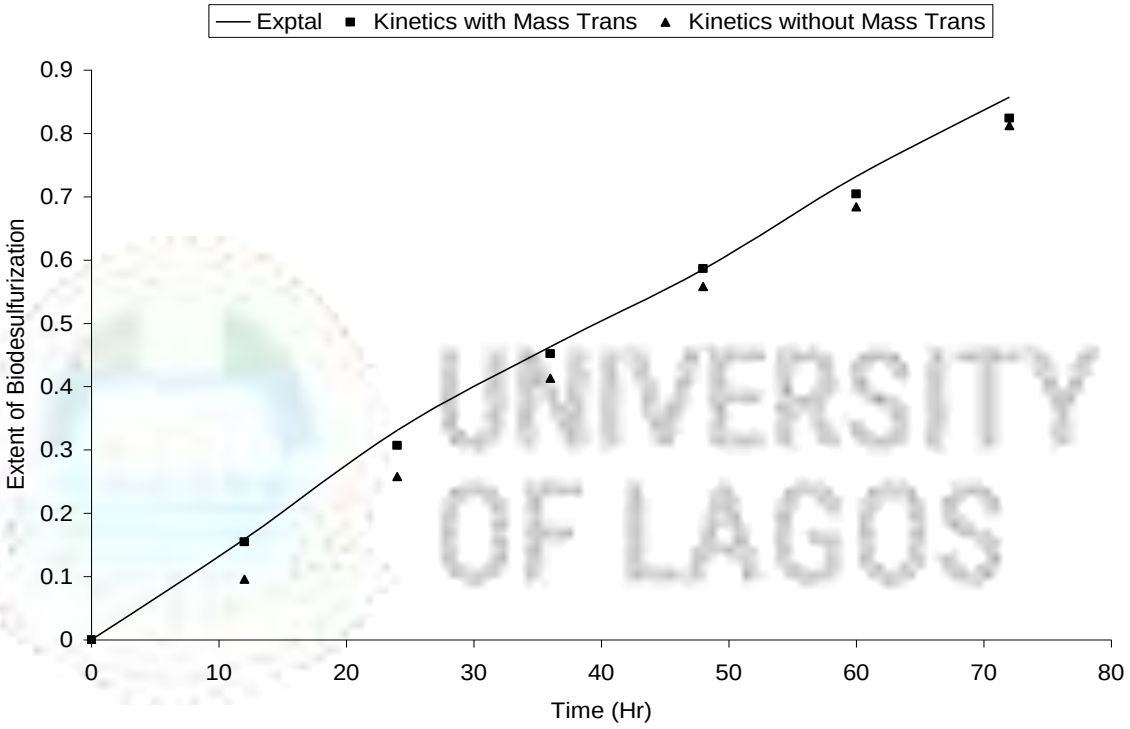


Figure 4.17: The Experimental and Simulated Conversion of Thiophene -Time Profile during Kerosene Biodesulfurization by *D. anilini*
(Kinetic Model without Mass Transfer and Mass Transfer Influenced Kinetic Model).

Similarly, Figure 4.18 showed the 2, 5 - Dimethylthiophene conversion – time profile in kerosene biodesulfurization. The deviations between the experimental and simulated data at the sample points range from 0.001 and 0.088 for kinetic model without mass transfer. The overall sum of the variances at these points is 1.735 x 10^{-2}. For the mass transfer influenced kinetic model the sum of variances of the simulated points compared to that of the experimental is 8.836 x 10^{-3} while the

deviation between the experimental and the simulated data points ranged from 0.008 to 0.070.

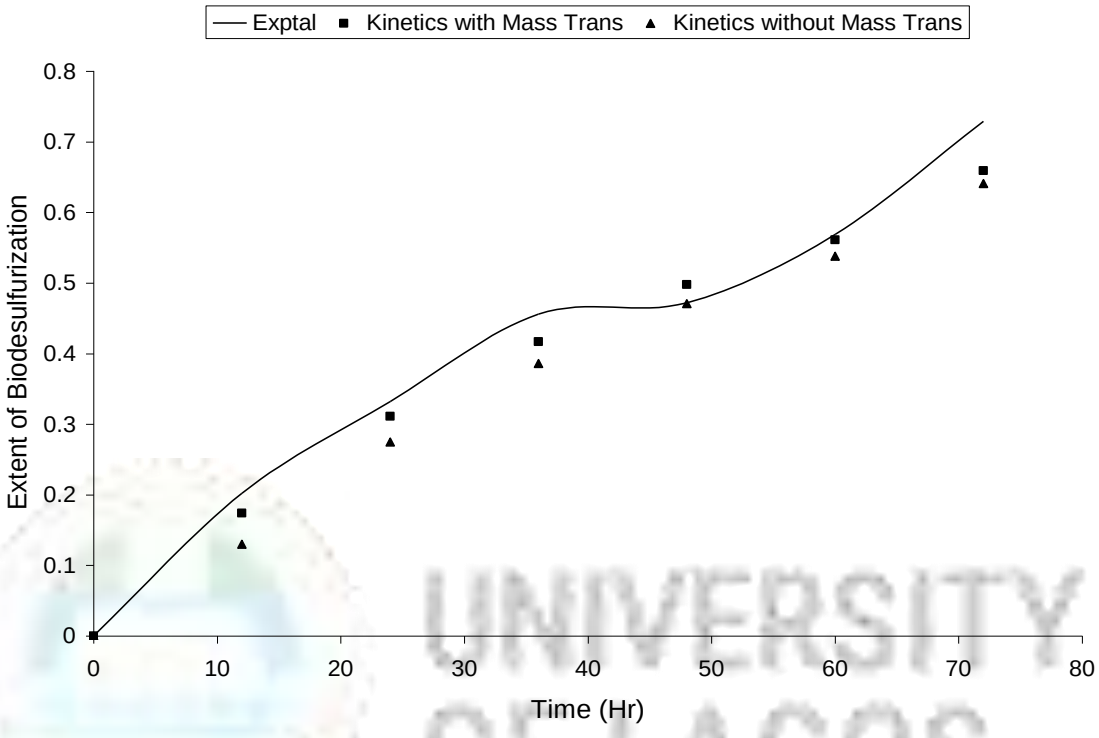


Figure 4.18: The Experimental and Simulated Conversion of 2, 5 - Dimethylthiophene -Time Profile during Kerosene Biodesulfurization by *D. anilini*
(Kinetic Model without Mass Transfer and Mass Transfer Influenced Kinetic Model).

It is clear from Figures 4.17 and 4.18 that the conversion – time profile for thiophene using the mass transfer influenced kinetic model during the biodesulfurization of kerosene by *Desulfobacterium anilini* showed the best agreement with the experimental data. This is followed by the 2, 5 - Dimethylthiophene conversion – time profile using the mass transfer influenced kinetic model by *Desulfobacterium anilini* then the profile of thiophene obtained from the kinetic model without mass transfer and lastly for 2, 5 – Dimethylthiophene profile from the kinetic model without mass transfer.

Also, Figures 4.19 and 4.20 showed the comparison of experimental and simulated conversion – time profiles during the biodesulfurization of kerosene by *Desulfobacterium indolicum* using kinetic model without mass transfer and mass transfer influenced kinetic model.

Figure 4.19 showed the thiophene conversion – time profile in kerosene biodesulfurization. The deviations between the experimental and simulated data at the sample points range from 0.028 and 0.072 for kinetic model without mass transfer. The overall sum of the variances at these points is 1.735×10^{-2}. For the mass transfer influenced kinetic model the sum of variances of the simulated points compared to that of the experimental is 5.254×10^{-3} while the deviation between the experimental and the simulated data points ranged from 0.002 to 0.057.

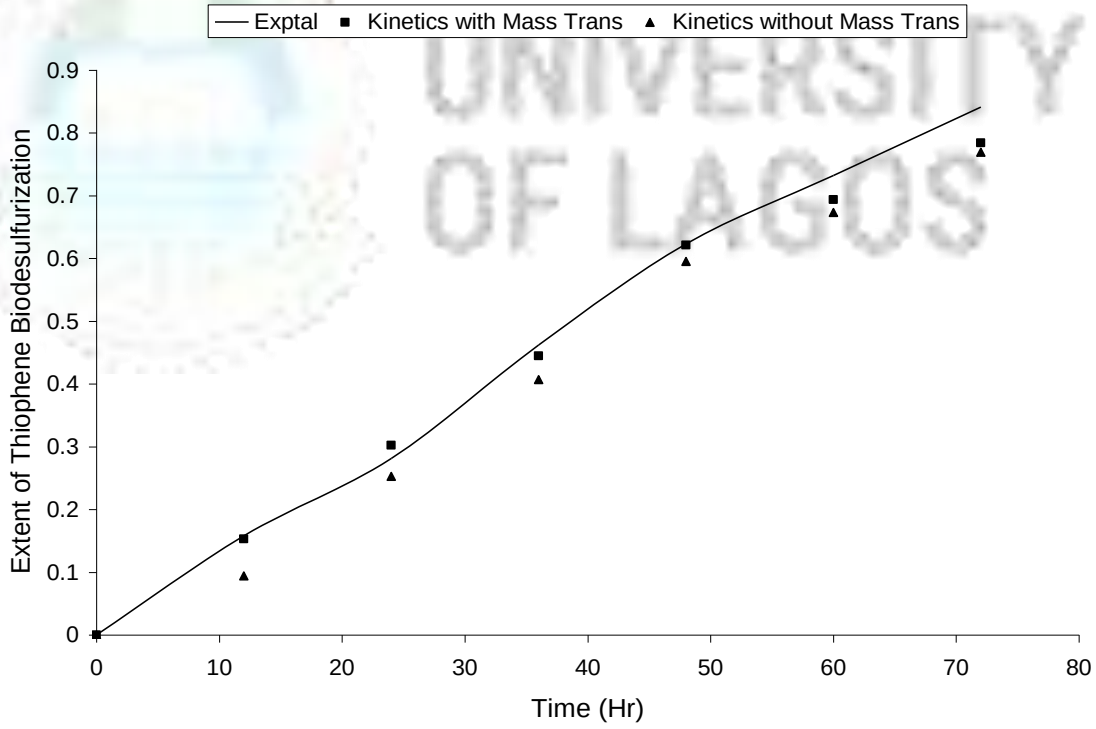


Figure 4.19: The Experimental and Simulated Conversion of Thiophene -Time Profile during Kerosene Biodesulfurization by *D. anilini*
(Kinetic Model without Mass Transfer and Mass Transfer Influenced Kinetic Model).

The extent of agreement of the 2, 5 – Dimethylthiophene conversion – time profile obtained from the mass transfer influenced kinetic model as well as that obtained from the kinetic model without the influence of mass transfer compared to the experimental data of biodesulfurization in kerosene by *Desulfobacterium indolicum* is shown in Figure 4.20. The sum of variance between the experimental and the2, 5 – Dimethylthiophene conversion – time profile obtained from the mass transfer influenced kinetic model is 1.118×10^{-2}, while the deviation of the data points vary from 0.003 to 0.086. For the kinetic model without the influence of mass transfer, the deviation of the data points vary from 0.030 to 0.106 while the sum of variance is 2.569×10^{-2}

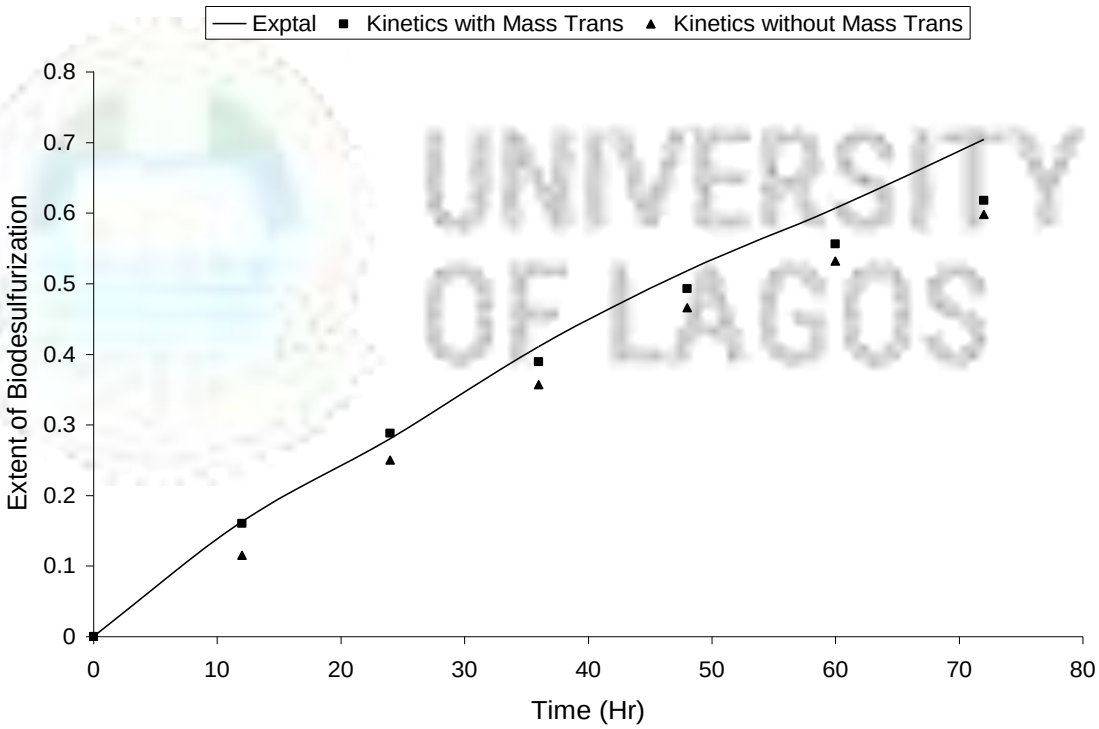


Figure 4.20: The Experimental and Simulated Conversion of 2, 5 - Dimethylthiophene -Time Profile during Kerosene Biodesulfurization by *D. indolicum*
(Kinetic Model without Mass Transfer and Mass Transfer Influenced Kinetic Model).

It can also be seen from Figures 4.19 and 4.20 that the conversion – time profile for thiophene using the mass transfer influenced kinetic model during the biodesulfurization of kerosene by *Desulfobacterium indolicum* showed the best

agreement with the experimental data. This is followed by the 2, 5 – Dimethylthiophene conversion – time profile using the mass transfer influenced kinetic model by *Desulfobacterium indolicum* then the profile of thiophene obtained from the kinetic model without mass transfer and lastly for 2, 5 – Dimethylthiophene profile from the kinetic model without mass transfer. This trend is similar to that obtained from the simulated data obtained from the biodesulfurization of kerosene by *Desulfobacterium anilini.*

Generally for the simulation of biodesulfurization of kerosene by *Desulfobacterium anilini* (Figures 4.17 and 4.18) and *Desulfobacterium indolicum* (Figures 4.19 and 4.20), the sum of variances range from 2.587×10^{-3} to 2.569×10^{-2} . The deviations from points also range from 0.001 to 0.106. One may then conclude that the simulated data for thiophene conversion – time profile obtained from mass transfer influenced kinetic model during kerosene biodesulfurization by *Desulfobacterium anilini* is best while the best data point obtained from the kinetic model is 0.001 from the same profile.

Figures 4.21 and 4.22 showed the comparison of experimental and simulated conversion – time profiles during the biodesulfurization of diesel by *Desulfobacterium anilini* using kinetic model without mass transfer and mass transfer influenced kinetic model.

Figure 4.21 showed the Dibenzothiophene conversion – time profiles using kinetic model without mass transfer and mass transfer influenced kinetic model in comparison with the experimental data during biodesulfurization of diesel by *Desulfobacterium anilini*. The sum of variances is 1.317×10^{-2} and the deviations between the experimental and simulated data at the sample points range from 0.030 to 0.076 for kinetic model without mass transfer while that for the mass transfer influenced kinetic model range from 0.005 to 0.044 and its sum of variance between the data points of the simulated and experimental is 3.854×10^{-3}.

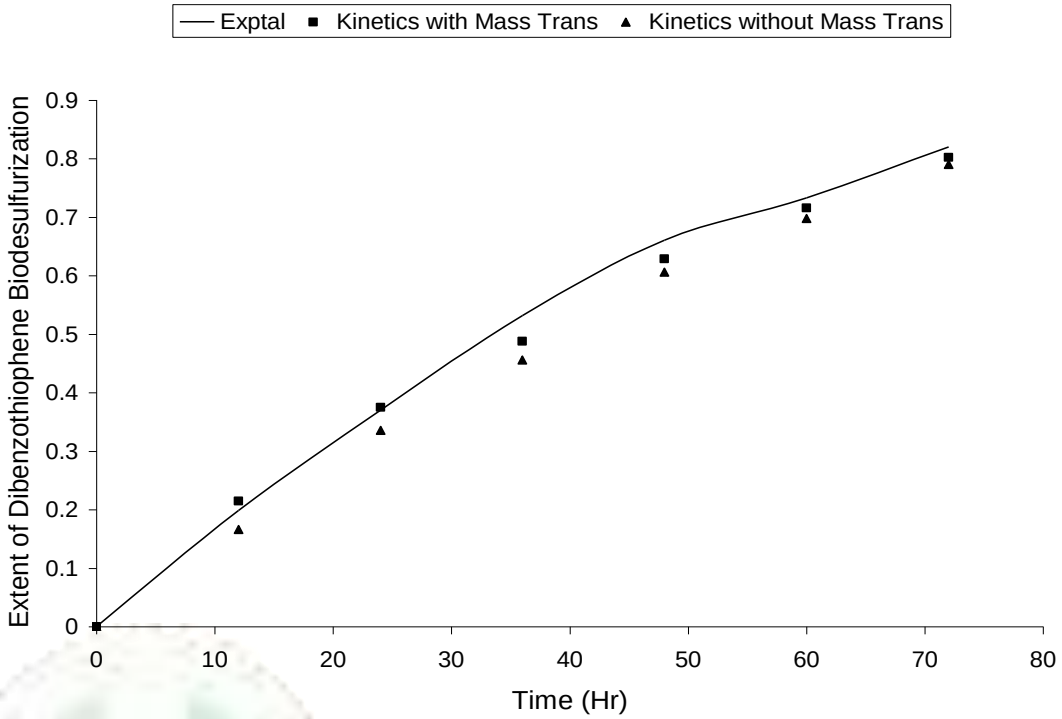


Figure 4.21: The Experimental and Simulated Conversion of Dibenzothiophene -Time Profile during Diesel Biodesulfurization by *D. anilini*
(Kinetic Model without Mass Transfer and Mass Transfer Influenced Kinetic Model).

Figure 4.22 showed the benzothiophene conversion – time profile during biodesulfurization of diesel. The comparison of the simulated data with the experimental data gave a sum of variances of 6.566 x 10^{-3} with the data points ranging from 0.009 to 0.047 when the kinetic model without mass transfer was used. For the mass transfer influenced kinetic model, the sum of variance between experimental and the simulated is 2.923 x 10^{-3} while the deviation at the data point range from 0.003 to 0.033.

It is obvious from Figures 4.21 and 4.22 that the conversion – time profile for benzothiophene using the mass transfer influenced kinetic model during the biodesulfurization of diesel by *Desulfobacterium anilini* showed the best agreement with the experimental data. This is followed by the Dibenzothiophene conversion – time profile using the mass transfer influenced kinetic model by *Desulfobacterium anilini* then the profile of benzothiophene obtained from the kinetic model without

mass transfer and lastly for Dibenzothiophene profile from the kinetic model without mass transfer.

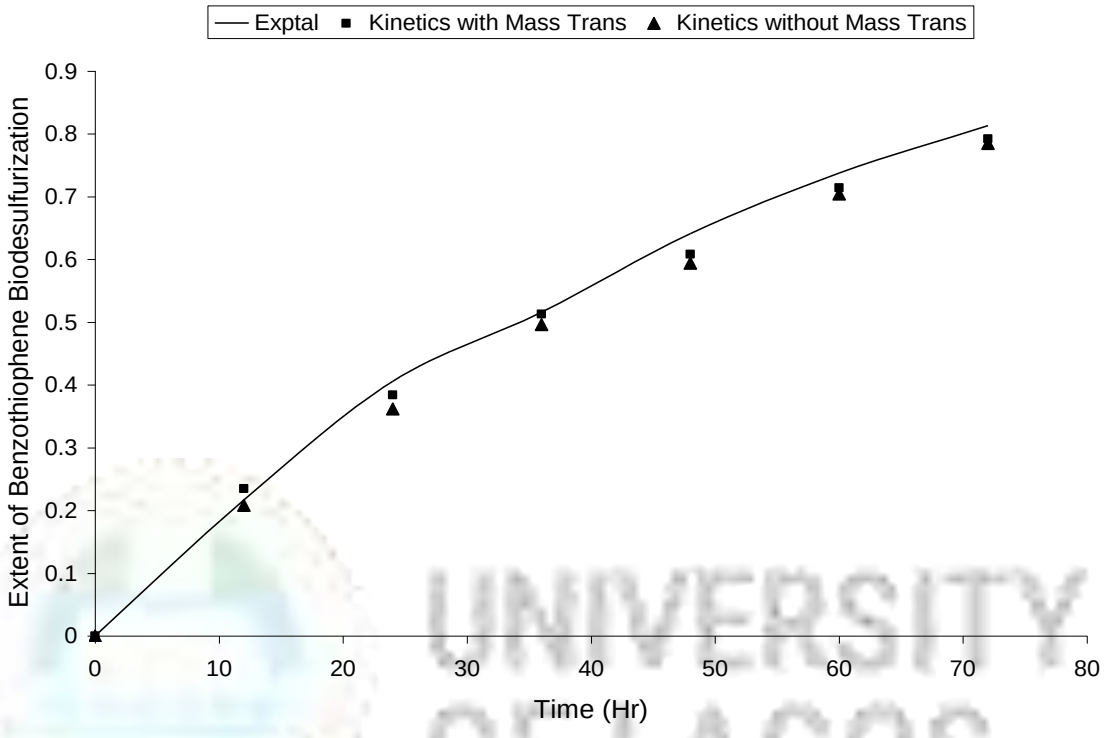


Figure 4.22: The Experimental and Simulated Conversion of Benzothiophene -Time Profile during Diesel Biodesulfurization by *D. anilini*
(Kinetic Model without Mass Transfer and Mass Transfer Influenced Kinetic Model).

Similarly, this study also provides the simulated data of the mass transfer influenced kinetics as well as the kinetic model without mass transfer compared to the experimental data of biodesulfurization of sulfur-containing hydrocarbon in diesel by *Desulfobacterium indolicum*. The profiles are shown in Figures 4.23 and 4.24.

Figure 4.23 showed the data of Dibenzothiophene conversion – time profiles. The sum of variance of the simulated mass transfer driven kinetic data from the experimental data gave a value of 1.096×10^{-3}. The least deviated point ranged from 0.001 to 0.022. The deviations between the experimental and simulated data at the sample points range from 0.007 and 0.052 for the kinetic model without mass transfer. The overall sum of the variances at these points is 4.539×10^{-3}.

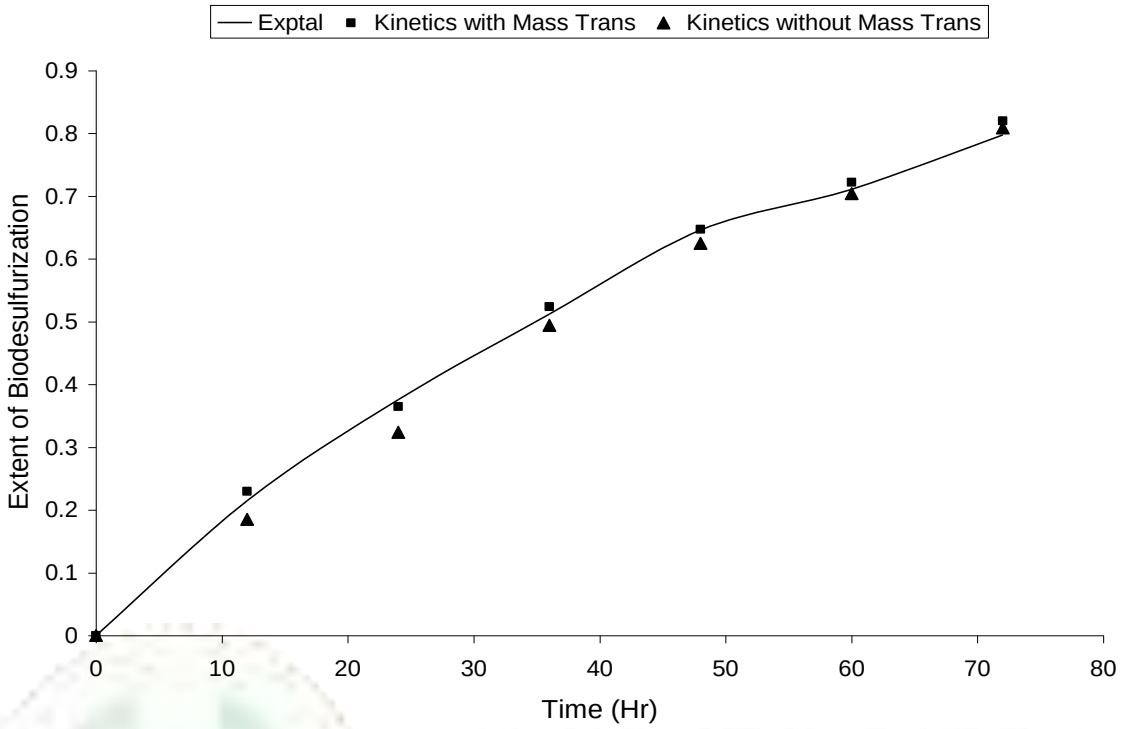


Figure 4.23: The Experimental and Simulated Conversion of Dibenzothiophene -Time Profile during Diesel Biodesulfurization by *D. indolicum*
(Kinetic Model without Mass Transfer and Mass Transfer Influenced Kinetic Model).

Figure 4.24 showed the profiles of benzothiophene conversion – time using the mass transfer driven kinetic model as well as kinetic model without mass transfer compared to the experimental data during the biodesulfurization of diesel by *Desulfobacterium indolicum*. The obtained value for the sum of variance is 1.187×10^{-2} while the deviation at the various point range from 0.003 to 0.860 with the mass transfer driven kinetic profile. For the kinetic model without mass transfer profile, the deviations range from 0.017 to 0.084 and the overall sum of the variances at these points is 1.849×10^{-2}.

From Figures 4.21 and 4.22, it is obvious that the conversion – time profile for dibenzothiophene using the mass transfer influenced kinetic model during the biodesulfurization of diesel by *Desulfobacterium indolicum* showed the best agreement with the experimental data. This is followed by the dibenzothiophene conversion – time profile using the kinetic model without mass transfer influence,

then the profile of benzothiophene obtained from the mass transfer influenced kinetic model and lastly for benzothiophene profile from the kinetic model without mass transfer.

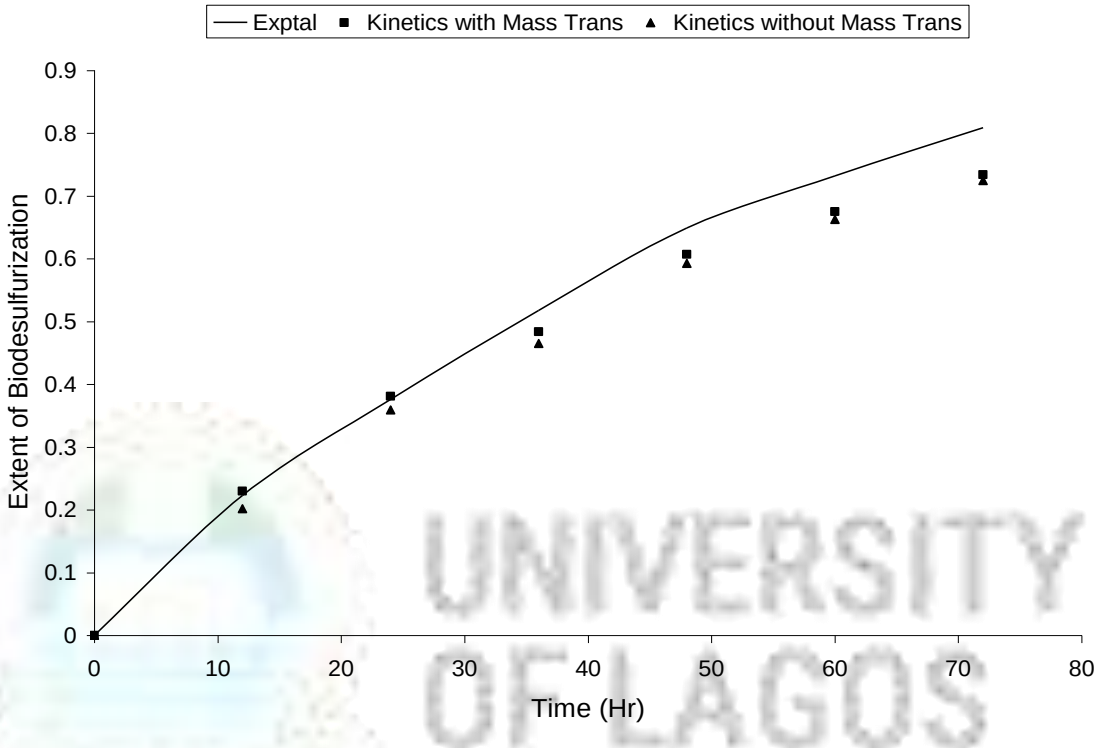


Figure 4.24: The Experimental and Simulated Conversion of Benzothiophene -Time Profile during Diesel Biodesulfurization by *D. indolicum*
(Kinetic Model without Mass Transfer and Mass Transfer Influenced Kinetic Model).

Generally for the simulation of biodesulfurization of diesel by *Desulfobacterium anilini* (Figures 4.21 and 4.22) and *Desulfobacterium indolicum* (Figures 4.23 and 4.24), the sum of variances range from 1.096×10^{-3} to 1.849×10^{-2}. The deviations from points also range from 0.001 to 0.084. One may then conclude that the simulated data for dibenzothiophene conversion – time profile obtained from mass transfer influenced kinetic model during kerosene biodesulfurization by *Desulfobacterium anilini* is best while the best data point obtained from the kinetic model is 0.001 from the same profile.

CHAPTER 5

5.1 DISCUSSION

The experimental and modeling results of the biodesulfurization of kerosene and diesel by *Desulfobacterium anilini* and *Desulfobacterium indolicum* are discussed in this chapter.

The cleavage of carbon-sulfur bonds can be performed either with an aerobic or an anaerobic mechanism. Based on the bond strengths summarized in Table 2.3, the C-S bonds in the sulfur heterocycles (thiophene, benzo- and dibenzothiophene) will be broken preferentially (Bressler *et al.,*, 1998) as the C-C bond strengths are greater than the C-S bond strengths. As can be seen from Table 2.3, the addition of oxygen to a carbon atom adjacent to the sulfur atom weakens the bond strengths making the C-S bond more susceptible to cleavage. This is a common feature in the aerobic microbial conversion of sulphur compounds, where enzymes (dioxygenases) introduce oxygen molecules to facilitate C-S cleavage. According to the bond strengths (Table 2.3) the C-S bonds will also be attacked preferentially in the anaerobic reaction mechanism that is similar to the metal-catalyzed HDS reaction mechanism. The role of enzymes to enable or support an attack on the C-S bond in the anaerobic route is currently not well understood.

A vast amount of research is performed on the development of aerobic microbiological desulfurization. Dibenzothiophene (DBT) is the key heterocylic sulfur compound used in most biodesulfurization studies. One of the aerobic pathways is the Kodama. The transformation of DBT in the 'Kodama' pathway results in ring cleavage of one of the aromatic DBT rings, while the sulfur is not released. The organisms perform an aspecific pathway using DBT as the sole source of carbon, sulfur and energy (Van Afferden *et al.,*, 1990). Owing to the degradation of C-C bonds the caloric value is altered, consequently this route is not desirable in biodesulfurization. The other pathway is sulfur selective which involves a stepwise selective oxidation of the hetero sulphur atom, while the carbon skeleton is not

metabolized. In particular sulfur is removed from DBT to give the end-product 2-hydroxybiphenyl (Gallagher *et al.,*, 1993). In contrast to aerobic biodesulfurization, evidence for the anaerobic conversion of organic sulfur compounds is equivocal. Kim *et al.,*, (1995) reported significant conversion of various model compounds under anaerobic conditions, accompanied with the concomitant formation of H_2S. Biphenyl was found as the major reaction product when dibenzothiophene was reductively converted. During the reductive conversion, DBT is used as the sole electron acceptor and sulfur is removed selectively (Kim *et al*., 1990).

The anaerobic route is a potentially attractive biodesulfurization route to apply, because of its sulfur specificity. From Figure 2.12, it follows that the caloric value is maintained because C – C bonds are not altered. Furthermore, the reaction pattern is similar to HDS. However, growth under anaerobic conditions proceeds slowly, especially when organic molecules (like thiophenes) are involved in the conversion. When thiophenes are used as the sole electron acceptor, the conversion of thiophenes should be coupled to microbial growth. From a process point of view, the aerobic route has some major drawbacks. Sulfur is used in the assimilatory metabolism of aerobic bacteria. Considering that the sulfur content of biomass is approximately 0.03 wt%, the yield of biomass per mole sulfur removed in the aerobic route is high. Approximately 50% of the energy produced by aerobic microorganisms will be used for growth, while anaerobic microorganisms use approximately 10% of their energy for assimilation (Marcelis, 2002). At high biomass concentrations downstream processing is complicated, because proteins originating from the biomass emulsify the oil/water mixture. In addition the mixing efficiency and O_2 availability is less optimal in emulsions with a high biomass concentration. Furthermore, diluted sulfate is formed as the end-product of the aerobic route that also must be removed, while H_2S that is formed in the anaerobic route can be treated with existing refinery desulfurization plants. In the aerobic sulfur specific route oxygen molecules are added to the hydrocarbon skeleton. This is not desirable, because 2-hydroxybiphenyl is involved in the formation of viscous oil sludge (’gum’) in the fuel. Furthermore, product inhibition by 2-hydroxybiphenyl might play a role. The 2-

hydroxybiphenyl formed in the cells eventually will diffuse back into the oil phase, but the phenolic molecule is known as a potent biocide. Based on the aforementioned considerations, the anaerobic route is chosen in this thesis.

Two microorganisms labeled A and D were isolated and subsequently subjected to various biochemical and morphological tests. The isolates were obtained anaerobically on a selective sulfur-reducing enrichment culture. The two isolates are rod-shaped, Gram-negative, and non-spore forming organisms. They utilize sodium lactate very poorly and are both positive to the hydrogen sulfide test. However, they tested negatively to urease, citrate, catalase and the Methyl Red-Voges Prausker (MRVP) tests. These results show that they do not contain the enzyme urease responsible for hydrolyzing urea to ammonia thereby raising the pH of their environment. Also, they do not contain the enzyme catalase that enables microorganisms break down hydrogen peroxide to form oxygen and water. The isolates had negative test to MRVP which is a characteristic feature of sulfur-utilizing bacteria as they are inactive at a pH of less than 4.5. The two isolates were also found to have the capability of utilizing glucose, mannitol and maltose. They also produced gas from glucose and utilized sodium propionate. Isolate A was tentatively identified as *Desulfobacterium indolicum* while isolate D as *Desulfobacterium anilini* based on their differential morphological and biochemical characteristics (Table 4.1) and by reference to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). They belong to the same family of sulfur-utilizing bacteria, *Desulfobacterium* but of different species based on this difference in their responses to biochemical tests.

In the course of the kinetic model development, enzyme catalyzed reactions were viewed as a heterogeneous catalytic reaction. Thus, the Boudart approach to heterogeneous catalysis was applied to enzyme catalysis (Susu, 1997). The result obtained showed that they are indeed one and that the order of reaction followed by such reaction depends on the step that determines the reaction. When the first step

(determines the rate, the formation of the enzyme-substrate complex from the reaction of the enzyme and substrate), the reaction is first order. On the other hand, the reaction follows the Michaelis-Menten equation when the second step (conversion of the enzyme-substrate complex to the product) limits the rate of reaction. An enzyme-catalyzed reaction may still follow the zero order. The order of reaction followed by a reaction must be determined from the available experimental data.

Furthermore, two types of kinetic models for the biodesulfurization of diesel and kerosene were developed. These are one in which the effect of mass transfer was considered and the other its effect was neglected. It is important to mention that the growth kinetics of the organisms was neglected. This is because the population densities of the organisms did not increase significantly during the biodesulfurization experiments as reflected by the optical density measurements (Initial value, 0.930 and final value, 0.934 at a wavelength, λ of 510 nm for *Desulfobacterium indolicum* and by standard plate counts (Initial value, 6.40 x 10^6 and final value, 6.43 x 10^6 cfu/ml) while for *Desulfobacterium anilini* the Initial value, 0.930 and final value, 0.936 at a wavelength, λ of 510 nm and the standard plate counts (Initial value, 6.40 x 10^6 and final value, 6.44 x 10^6 cfu/ml). It is plausible to say that the sulfur compounds were probably utilized by the organisms to synthesize some amino acids such as methionine and cysteine required for sustenance and not necessarily for procreation. The developed kinetic models were first order ordinary differential equations whose analytical solution resulted in implicit equations (Equations 3.50 and 3.64). Hence, a numerical approach was used to solve the expressions. The implicit Finite Difference method was used because it is accurate, consistent and stable. Implicit equations are generally difficult to solve, but their solution by Finite Difference method is not restricted by stability criteria. There is also no restriction on the size of the time step, Δt (Kaw and Kalu, 2008).

For the biodesulfurization experiment, the organisms *Desulfobacterium indolicum* and *Desulfobacterium anilini* were suspended in sulfur-free phosphate buffer medium of

pH 7 and the fuel (diesel and kerosene) to which 2% glucose was added to serve as a source of carbon for the organisms. It is easier for the organism to utilize carbon in glucose which is in aqueous state in which the organism is also suspended if available than kerosene which is oil. The cells were observed at the interface of the aqueous and oil phase after the reaction broth was centrifuged. This suggests that they did not secrete surfactants that could have broken down the substrates prior to biodesulfurization by the organisms. Instead, the organisms increased their cell surface hydrophobicity so that adherent capacity of the hydrocarbon was enhanced thus pointing to the fact that the substrates have not been broken down. The enzymes responsible for the biodesulfurization are described as being extracellular, that is the enzymes are secreted to the surface of the organisms by stimulus and thereafter act on the substrates. This is the reason why the structures of the substrate will remain intact even when the sulfur atom has been abstracted.

Several researchers, including Oldfield *et al.,* (1997) and Monticello (2000) , have reported that the desulfurization reactions occur within the cells. In *R. erythropolis* IGTS8, Dsz enzymes are soluble and presumably found in the cytoplasm (Gray *et al.,*, 1996). While the intracellular metabolism of DBT by strain IGTS8 has been reported, there is no evidence that DBT is actively transported into the cell (Monticello, 2000), nor is there evidence for mass transfer limitations in DBT metabolism (Kilbane and Le Borgne, 2004). It has been reported that in strain IGTS8, desulfurization activity was associated with the external surface of the cells (Kayser *et al.,*, 1993 ; Kilbane, 1991; Patel *et al.,*, 1997). A substantial proportion (70 %) of the total desulfurization activity expressed by this strain was found in the cell debris fraction, which contains external cell membrane and cell-wall fragments; thus, in this organism, the enzyme biocatalyst responsible for desulfurization was reported as being a component of the cell envelope (Monticello, 1996). There is no evidence that Dsz enzymes are excreted from IGTS8 cells, but the size of substrates metabolized and the ability of other bacterial species to successfully compete for sulfur liberated from organosulfur substrates by strain IGTS8 make it likely that desulfurization does not occur intracellularly, but in association with the external surface of cells (Kilbane

and Le Borgne, 2004). Therefore, it is believed that the desulfurization pathway may function in association with the cell membrane, such that extracellular substrates and intracellular cofactors can both be accessed.

Figures in Appendix A showed the chromatograms of the diesel and kerosene before and 12, 24, 36, 48, 60, 72 hours after the biodesulfurization reaction by *Desulfobacterium anilini* and *Desulfobacterium indolicum.* The GC analysis revealed that the kerosene contained 6.955 mg/l of thiophene and 41.724 mg/l of 2, 5 – dimethyl thiophene and no benzothiophene and Dibenzothiophene were found in it. Similarly, diesel was found to contain 9.006 mg/l of benzothiophene and 157.031 mg/l of Dibenzothiophene but no thiophene and 2, 5 – dimethyl thiophene were detected in it. Figures A.1 and A.2 showed the chromatograms of kerosene and diesel respectively before the desulfurization experiments. The GC-PFPD peaks for all of the sulfur compounds in the kerosene (approximately 48.679 mg/l sulfur initially) before desulfurization is shown in Figure A.1. After treatment of the kerosene by contacting it with *Desulfobacterium indolicum* and *Desulfobacterium anilini* for 72 hours, all of the peaks significantly decreased (Figures A.26 and A.20). Similarly, the GC-PFPD peaks for all of the sulfur compounds in the diesel (approximately 166.037 mg/l sulfur initially) before the biodesulfurization by *Desulfobacterium indolicum* and *Desulfobacterium anilini* is shown in Figure A.2, after treatment of the Diesel by contacting it with *Desulfobacterium indolicum* and *Desulfobacterium anilini* for 72 hours, all of the peaks significantly decreased, these chromatograms are shown in Figures A.14 and A.8. Figures A.3 to A.26 showed that the sulfur compounds with retention times longer than 5 minutes nearly disappeared. Such characteristics of desulfurization by cells of *Desulfobacterium anilini* and *Desulfobacterium indolicum* are opposite or complimentary to those of hydrodesulfurization, in which sulfur compounds with a shorter residence time are more easily desulfurized (Dzidic *et al*, 1988). Based on these results, cells of *Desulfobacterium anilini* and *Desulfobacterium indolicum* are considered to have a sufficiently broad substrate specificity to desulfurize major organic sulfur compounds contained in diesel.

All the experimental data were found to be statistically significant at 95% confidence level. The student's t-test statistical tool was used for the tests. The detailed results of the test is in appendix B. The extent of biodesulfurization-time profiles of the sulfur-containing organic compounds in kerosene by *Desulfobacterium anilini* and *Desulfobacterium indolicum* are shown in Figures 4.1 to 4.4. Figures 4.1 and 4.3 show the pattern of thiophene biodesulfurization in kerosene by *D anilini* and *D indolicum* respectively. It is instructive to note that the occurrence of relatively low value of K_M to the initial concentration of the thiophene is an indication of a zero order reaction. This is evident in the almost linear profile of the biodesulfurization. When this happens, one may conclude that the rate at which the substrate gets to the organism does not meet its demand (Atlas, 1985). The relative low rate of biodesulfurization of the 2, 5-Dimethylthiophene compared to that of thiophene may be attributed to steric hindrances caused by the two methyl groups attached to the thiophene ring. The steric factor will limit access of the microorganisms to the substrate, thus affecting the rate of the sulfur removal. Several authors have reported the difficulty in desulfurizing model compounds with substituents attached to them (Li *et al*., 2003, Chang *et al*., 1998 and Kilbane, 2006). However, the initial concentrations cannot be considered to be negligible to the Michaelis-Menten constants nor can the Michaelis-Menten constants be considered negligible to the initial concentrations for 2, 5 dimethylthiophene, dibenzothiophene and benzothiophene. For this reason, the kinetics may not be first order or zero order but follow the Michaelis-Menten pattern. It can be seen that the profiles are not linear (Figures 4.2 and 4.4). Generally, *Desulfobacterium anilini* desulfurized kerosene better than *Desulfobacterium indolicum*.

Interestingly, the cells of *Desulfobacterium indolicum* and *Desulfobacterium anilini* were also found capable of significantly desulfurizing diesel. The profiles of biodesulfurization with time of the sulfur heterocycles are shown in Figures 4.5 to 4.8. These results are particularly interesting because most regulations are directed towards the sulfur content of diesel. Diesel contains mainly benzothiophenes and their various derivatives. The biodesulfurization of Dibenzothiophene (DBT) by *D*

anilini is shown in figure 4.5. This reaction is interesting because DBT is the model substrate that has been used in the studies of biodesulfurization over the years. The kinetics of the biodesulfurization of the sulfur-containing components of diesel followed the Michaelis-Menten pattern. It can be seen that the profiles are not linear (Figures 4.5 and 4.8). Comparative studies of the biodesulfurizing capability of the microorganisms revealed that the *Desulfobacterium anilini* performed better than *Desulfobacterium indolicum* even though the two microorganisms did well.

In this study, the kinetic models for the biodesulfurization of diesel and kerosene were developed. Figures 4.9 to 4.16 show the comparison for the kinetic models with the experimental data. The kinetic parameters used in the simulation were those obtained from the experimental data subjected to iterative nonlinear least-squares regression analysis method of Macquardt (Susu, 1997). The distribution coefficient was obtained from literature (Arey and Gschwend, 2005). The level of agreement between the simulated and experimental data was determined by the sum of variances between the sets of data. The sum that is lower between two sets of data has a better agreement.

The simulations of the kinetic model of kerosene biodesulfurization by *Desulfobacterium anilini* are shown in Figures 4.9 and 4.10. The data obtained from thiophene simulation gave a better agreement with the experimental data than for 2, 5 – dimethylthiophene because the sum of the variances of the thiophene profile is lower than that of 2, 5 – Dimethylthiophene. Similarly, the simulated kinetic model of the kerosene biodesulfurization by *Desulfobacterium indolicum* was also carried out. The obtained profiles compared with the experimental values are shown in Figures 4.11 and 4.12. The same pattern of agreement of the data in the *Desulfobacterium anilini*- kerosene system was observed.

Similarly, the kinetic model developed for the biodesulfurization of diesel by *Desulfobacterium anilini* and *Desulfobacterium indolicum* was also simulated. Figures 4.13 to 4.16 showed the obtained profiles compared with the profiles of the experimental results. The profiles exhibited by the desulfurization of the sulfur-containing organic compounds by *Desulfobacterium anilini* are shown in Figures 4.13

and 4.14 while those of *Desulfobacterium indolicum* were shown in Figures 4.15 and 4.16. It was observed that in the biodesulfurization of the sulfur compounds in diesel by the microorganisms, the simulated data of dibenzothiophene by *Desulfobacterium indolicum* fitted best into the experimental data, followed by the data of benzothiophene biodesulfurization by *Desulfobacterium anilini* then Dibenzothiophene by *anilini* and lastly the simulated data of benzothiophene by *Desulfobacterium indolicum*. The best fitted data point in the simulation of diesel biodesulfurization can be found for dibenzothiophene by *Desulfobacterium indolicum* at the 60^{th} hour with a difference of 0.007, the worst being that obtained at the 72^{nd} hour of benzothiophene biodesulfurization by *Desulfobacterium indolicum*. The difference was 0.084

The kinetics of chemical analyses of catalytic reactions without the incursion of transport processes is a normal practice for two main reasons. In the first place, if fundamental knowledge of a reaction is available through the mechanism of the reaction, it will enhance the chances of catalyst design that will take advantage of the optimal reaction pathways to the desired products. Secondly, the usual procedure for reactor design of an industrial process involves the coupling of the intrinsic rate kinetics of the reaction with the transport relationships. These relationships are important for industrial processes since any of the various diffusion processes may be the limiting factor(s).

Figures 4.17 to 4.24 showed the comparison of the experimental data with simulation results of kinetic models with and without mass transfer factors. Figures 4.17 to 4.20 show the profiles as it concerned the biodesulfurization of kerosene. The comparisons of the simulated data of the two kinetic models (mass transfer influenced kinetic model and the kinetic model without the influence of mass transfer) with the experimental data of biodesulfurization of kerosene by *Desulfobacterium anilini* were shown in Figures 4.17 and 4.18. The simulated data points of the mass transfer influenced kinetic model exhibited better agreement with the experimental data than those of the kinetic model without mass transfer in all cases. The same pattern of agreement was observed for the *Desulfobacterium indolicum*-kerosene

system shown in Figures 4.19 and 4.20. Its sum of variance is 2.857 x 10^{-3} followed by the profile of mass driven kinetics of thiophene in kerosene by *Desulfobacterium indolicum* then that of 2, 5 – dimethylthiophene biodesulfurization by *Desulfobacterium anilini* and lastly that of 2, 5 – dimethylthiophene biodesulfurization by *Desulfobacterium indolicum.* The least deviation of the data point is 0.001 at the 48th hour of thiophene biodesulfurization in kerosene by *Desulfobacterium anilini*. It was generally observed that the data of the simulated mass transfer influenced kinetics and those of the kinetics without mass transfer is getting closer as the biodesulfurization progresses. However due to the better agreement of the simulated data of the mass transfer influenced kinetic model with the experimental data than those of the kinetic model without the influence of mass transfer, one may infer that the kinetics of the biodesulfurization of kerosene is mass transfer driven.

Figures 4.21 to 4.24 showed comparison of the extent of biodesulfurization versus time profiles for the ordinary and mass transfer-controlled kinetic models with experimental data of diesel biodesulfurization by *Desulfobacterium anilini* and *Desulfobacterium indolicum*. It was generally observed that the extent of biodesulfurization with time profiles of kinetic with mass transfer model had better agreement with the experimental data than those of kinetic without mass transfer. This trend was observed for all the cases as shown in Figures 4.17 to 4.24. Based on the observed trends, one may infer that the kinetics of the biodesulfurization of sulfur-containing compounds in fuel is generally influenced by mass transfer factors.

It can be seen that the simulated substrate concentration versus time data for the biodesulfurization of diesel and kerosene showed excellent agreement with experimental data. This shows that for a significant biodesulfurization to take place the substrates must be available and the microorganisms on their own must be capable of carrying out the reaction. This means that the various assumptions made in the model development that the two isolated bacteria, *Desulfobacterium indolicum* and *Desulfobacterium anilini* desulfurized the diesel and kerosene by selectively removing the sulfur from the various organosulfur compounds in the fuel without any significant distortion in the carbon frameworks were reasonable. Furthermore, the cells of *Desulfobacterium indolicum* and *Desulfobacterium anilini* were observed at

the interface of the aqueous and oil phase after the reaction broth was centrifuged. This suggested that they did not probably secrete surfactants that could have broken down the substrates prior to assimilation by the organisms, thus pointing to the fact that the substrates have not been broken down (Goswami and Singh, 1991). The enzymes responsible for the biodesulfurization are described as being extracellular, that is the enzymes are secreted to the surface of the organisms by stimulus. Moreover, degradation of petroleum products such as diesel and kerosene is usually aerobic and, therefore requires extensive aeration and agitation to make available sufficient oxygen for the process. In contrast, the sulfur-reducing bacteria are anaerobic and therefore, could not be involved in breaking down the diesel and kerosene substrates which takes place under anaerobic conditions.

No matter the level to which the genes of the organisms are amplified, the substrates must be available for them to perform effectively. When the organism and the substrates are not in the same phase, effort must be made to make the substrate available. Mass transfer processes such as stirring and agitation have been shown to enhance the performance of microorganisms in bioreaction (Gupta *et al*., 2005)

5.2 SUMMARY OF FINDINGS

In this section, the summary of this research findings are enumerated.

♦ Two microorganisms that were isolated and subsequently identified presumptively as Desulfobacterium *anilini* and *Desulfobacterium indolicum, both* Sulfur-reducing Bacteria were found to have the capabilities of selectively desulfurizing sulfur-containing organic compounds in middle distillate petroleum fractions such as diesel and kerosene without reducing their carbon content.

Further, the population densities of the organisms were found not to have increased significantly after the biodesulfurization of the fuels.

The GC-PFPD peaks of all the sulfur compounds in diesel and kerosene were found to significantly decrease after the biodesulfurization of the fuels.

It was also found that at least 70% of the sulfur-containing organic compounds in the fuel were desulfurized.

Furthermore, *Desulfobacterium anilini* was found to have desulfurized the sulfur-containing organic compounds in the fuels better than *Desulfobacterium indolicum* at the end of 72 hours for all the cases considered.

♦ Enzyme-catalyzed reactions were found to be a type of heterogeneous catalytic reaction because the rate expression for enzyme-catalyzed reactions was obtained by the method outlined by Boudart for those of heterogeneous catalysis. The rate determining step was able to show that when the first step determines the rate (the formation of the enzyme-substrate complex from the reaction of the enzyme and substrate), the reaction is first order.

On the other hand, the reaction follows the Michaelis-Menten kind of equation when the second step (conversion of the enzyme-substrate complex to the product) limits the rate of reaction.

The necessity of carrying out a non-linear regression analysis on the data before they can be used for simulation was found necessary because the kinetic parameters obtained from the experimental data of the biodesulfurization by the various linear plots of Eadie-Hofstee, Hanes and Lineweaver-Buck were not the same.

♦ The simulated data of the mass transfer influenced kinetics was found to show better agreement with the experimental data better than those of kinetic model without mass transfer.

5.3 CONTRIBUTIONS TO KNOWLEDGE

The following contributions were made to knowledge in the present study.

i. Two microorganisms, namely *Desulfobacterium anilini* and *Desulfobacterium indolicum* were isolated and identified. These microorganisms were found to have the capability to selectively remove sulfur anaerobically from diesel and kerosene without destroying their carbon framework.

ii. The kinetics of biodesulfurization of diesel and kerosene were found to be influenced by mass transfer factors. The mass transfer influenced kinetic model was used to simulate the substrate concentration versus time data for the biodesulfurization of diesel and kerosene. The predicted substrate concentration versus time data showed excellent agreement with the experimental data for diesel and kerosene.

iii. This study showed in all cases, that *Desulfobacterium anilini* desulfurized diesel and kerosene better than *Desulfobacterium indolicum*.

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

This study has been able to isolate two microorganisms capable of selective removal of sulfur from real petroleum feeds through the reductive pathway. In addition, kinetic models were developed for biodesulfurization of these feeds. Further, the simulated models show very good agreement with the experimental data. The quantitative parameters (measured, calculated and estimated) represent a valuable pool of information that can be used for reactor designs that will take the technology to the market place. One may then conclude that the output of this research will be useful in taking the technology to the market place.

6.2 RECOMMENDATIONS

The sulfur-contaminants in fuel are highly hydrophobic and thus sparingly soluble in water. The microorganisms that will carry out the biodesulfurization are normally suspended in aqueous solution. The development of methods that will enhance the solubilities of the heterocycles without distorting their framework will go a long way in making the proposed method readily available.

In order to develop biodesulfurization, the interdisciplinary participation of experts in biotechnology, biochemistry, refining processes and chemical engineering will be essential. Over the last two decades several research groups have attempted to isolate and characterize bacteria capable of desulfurizing oil fractions. Further research into biodesulfurization development is required before realistic assessments in pilot-plant studies can be made. It will be difficult to be competitive with chemical desulfurization, which has also made a lot of progress recently.

For any process to be viable in the petroleum industry it must not only be capable of treating the complex mixture of chemicals that constitute petroleum but it must also treat very large volumes in a cost-effective way. The two main steps to the commercial success of biodesulfurization are;

(i) To continue making rapid technical progress, and

(ii) To find optimum ways to integrate biotechnology into the refineries.

Integrating a biodesulfurization process into a refinery is the only way to treat fuel, but this requires a substantial modification of current refinery operations. In addition, the biodesulfurization process must operate at the same speed and reliability as other refinery processes so as not to disrupt normal refining operations. Thus despite the great interest and potential of biodesulfurization, it will be challenging to develop it to a stage where it can be practically implemented in refineries.

In view of this, a pilot scale study of anaerobic biodesulfurization is recommended.

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APPENDIX A

GC-PFPD CHROMATOGRAMS OF SOME ORGANOSULFUR COMPOUNDS

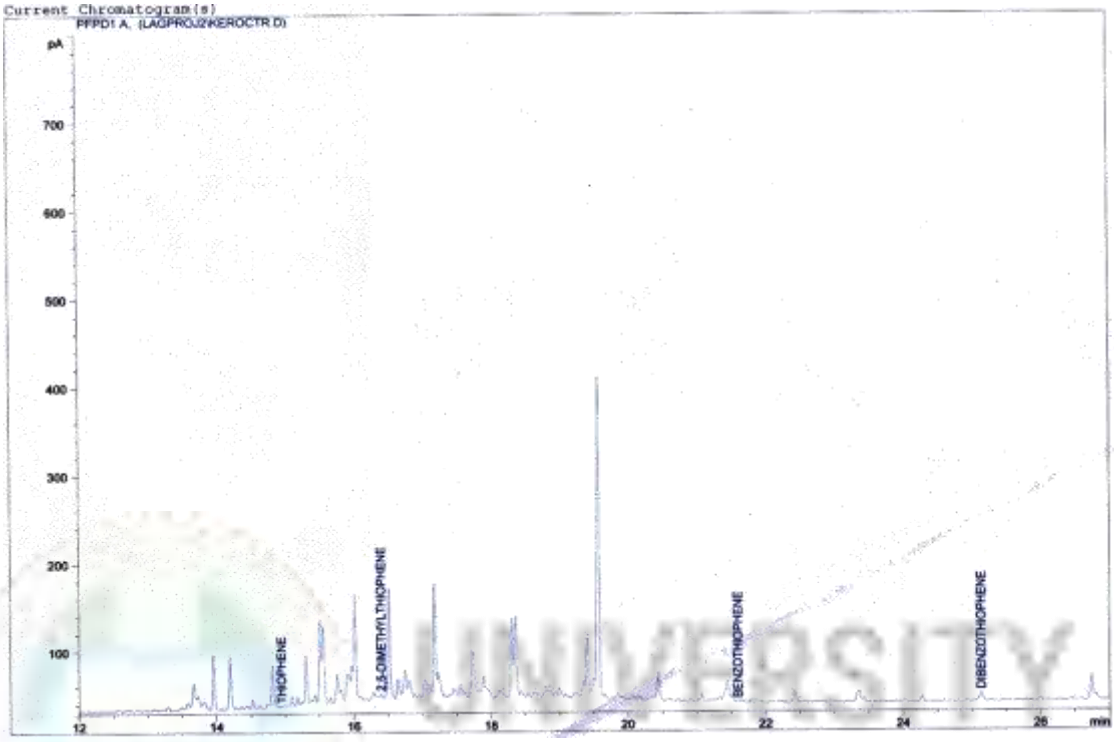

Figure A.1 GC-PFPD Chromatogram of the Kerosene before Biodesulfurization

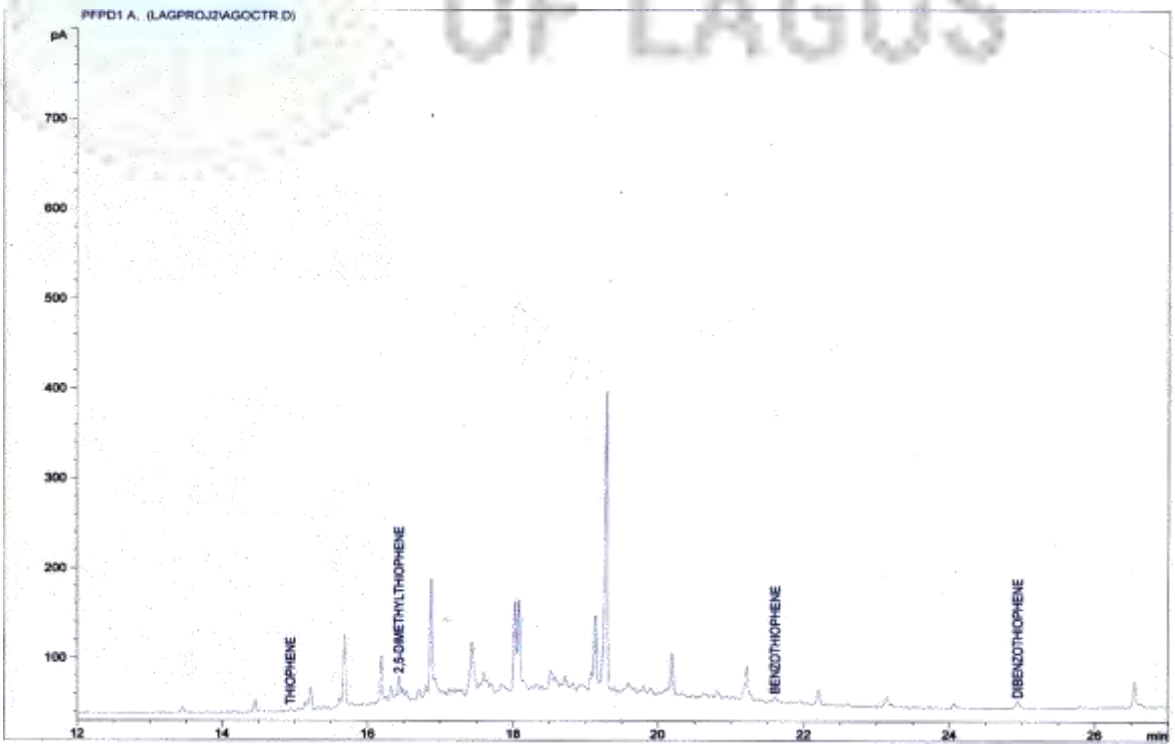

Figure A.2 GC-PFPD Chromatogram of the Diesel before Biodesulfurization

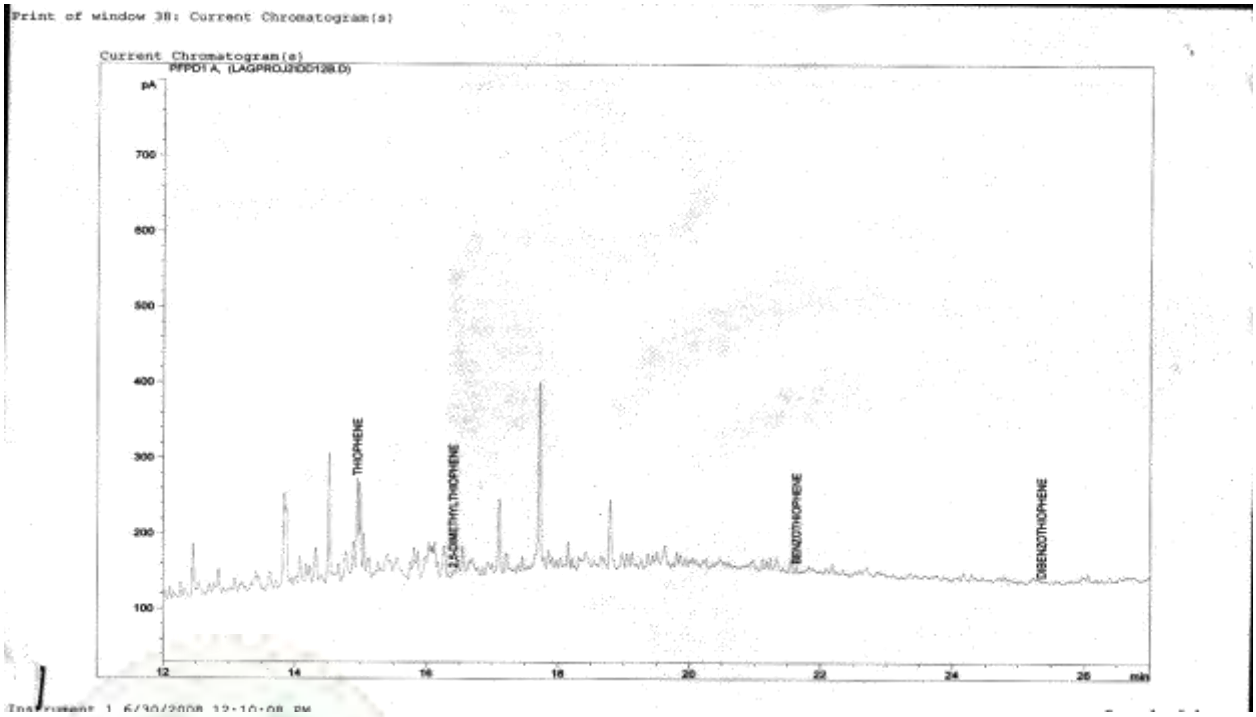

Figure A.3 GC-PFPD Chromatogram for Biodesulfurization of Diesel by *D anilini* at 12 Hours

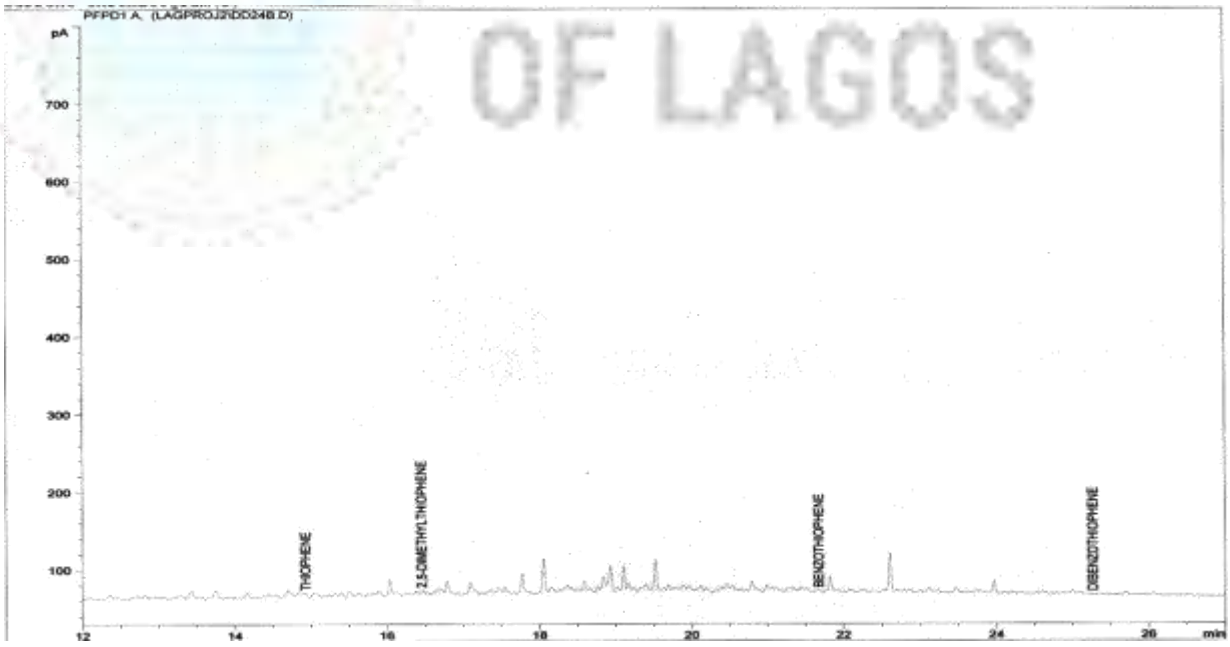

Figure A.4 GC-PFPD Chromatogram for Biodesulfurization of Diesel by *D anilini* at 24 Hours

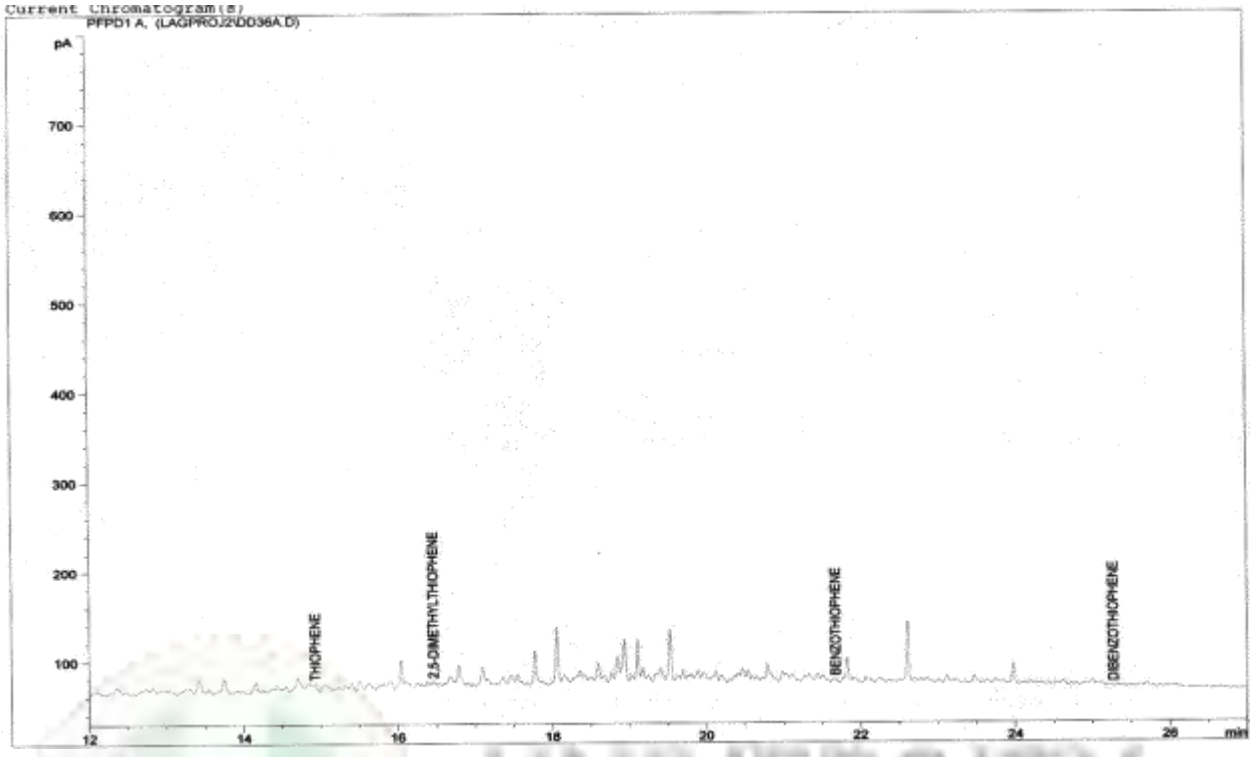

Figure A.5 GC-PFPD Chromatogram for Biodesulfurization of Diesel by *D anilini* at 36 Hours

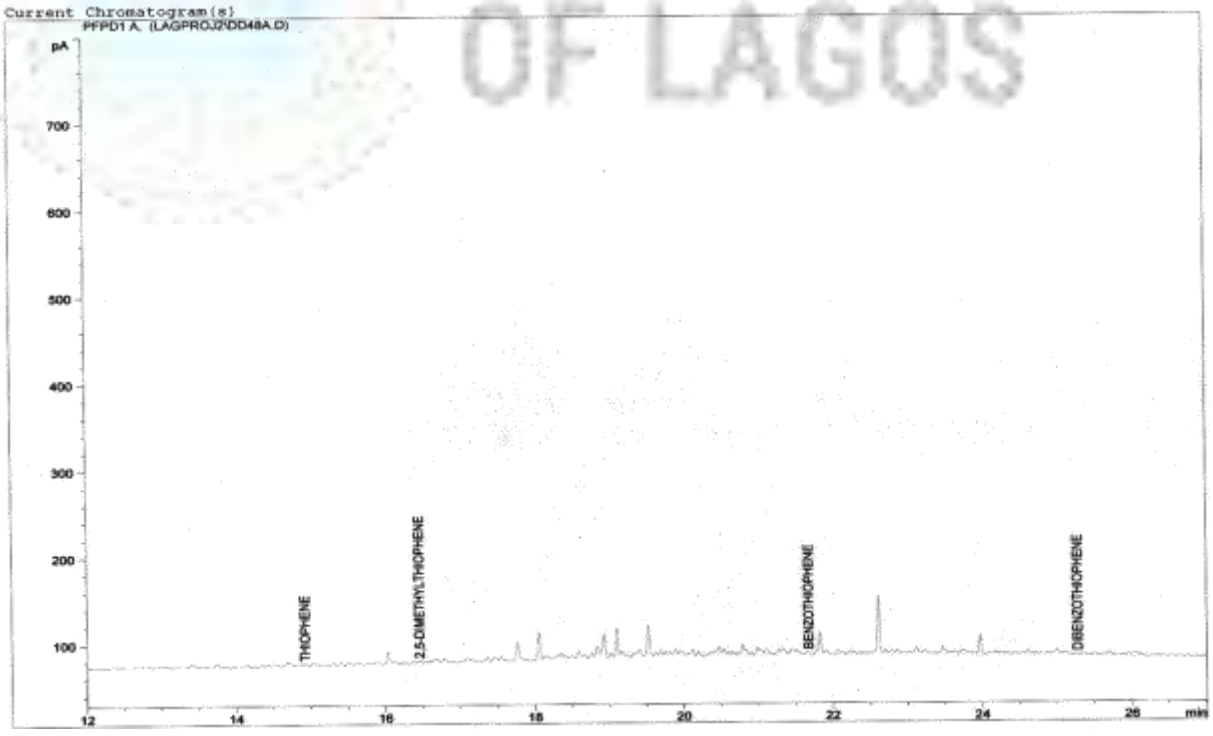

Figure A.6 GC-PFPD Chromatogram for Biodesulfurization of Diesel by *D anilini* at 48 Hours

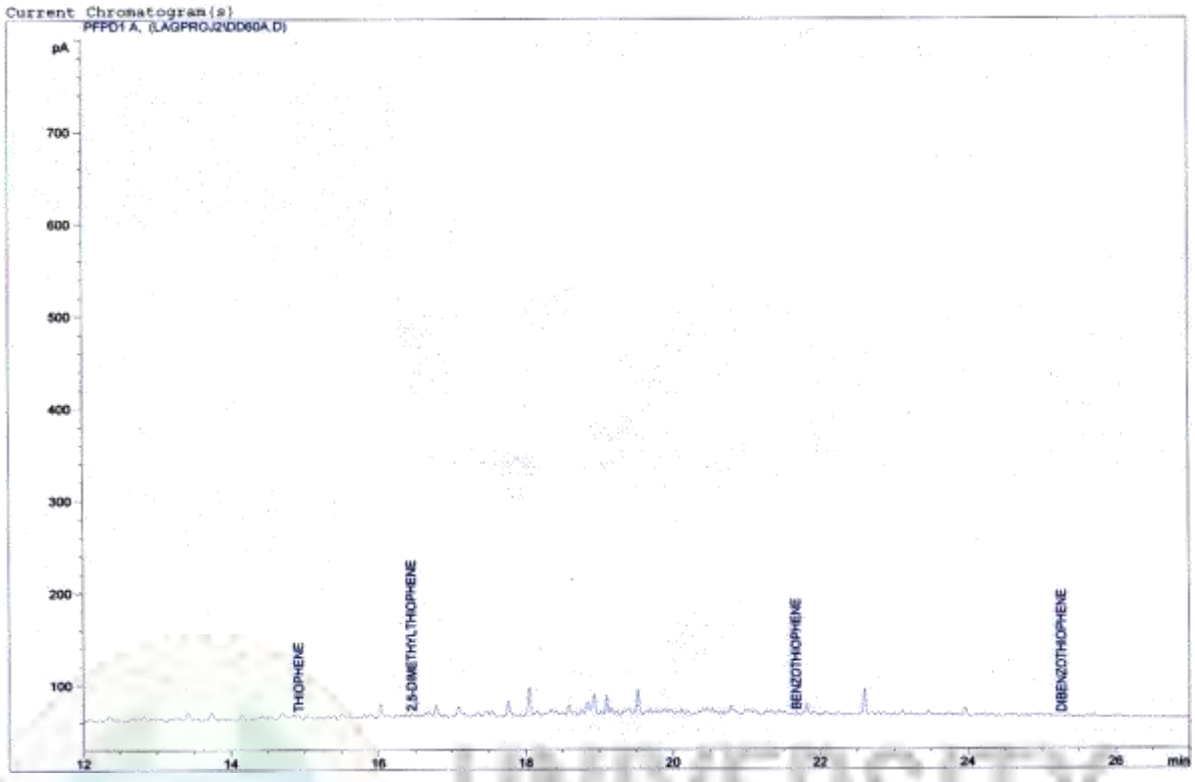

Figure A.7 GC-PFPD Chromatogram for Biodesulfurization of Diesel by *D anilini* at 60 Hours

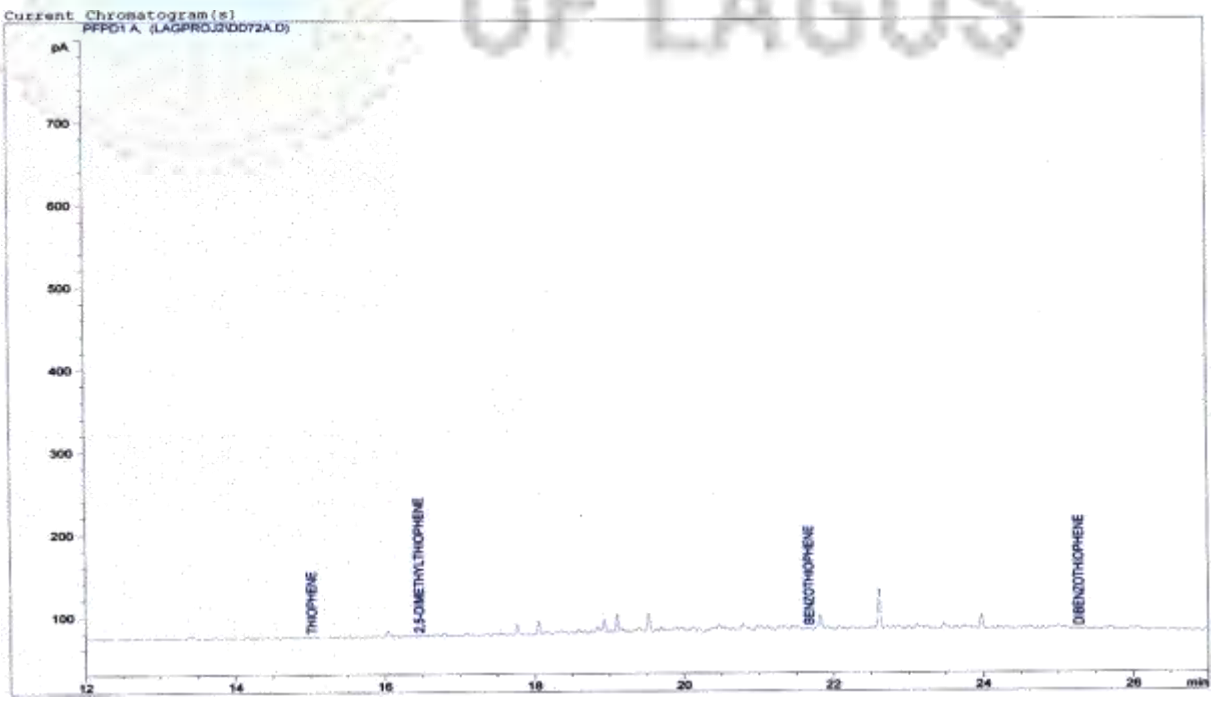

Figure A.8 GC-PFPD Chromatogram for Biodesulfurization of Diesel by *D anilini* at 72 Hours

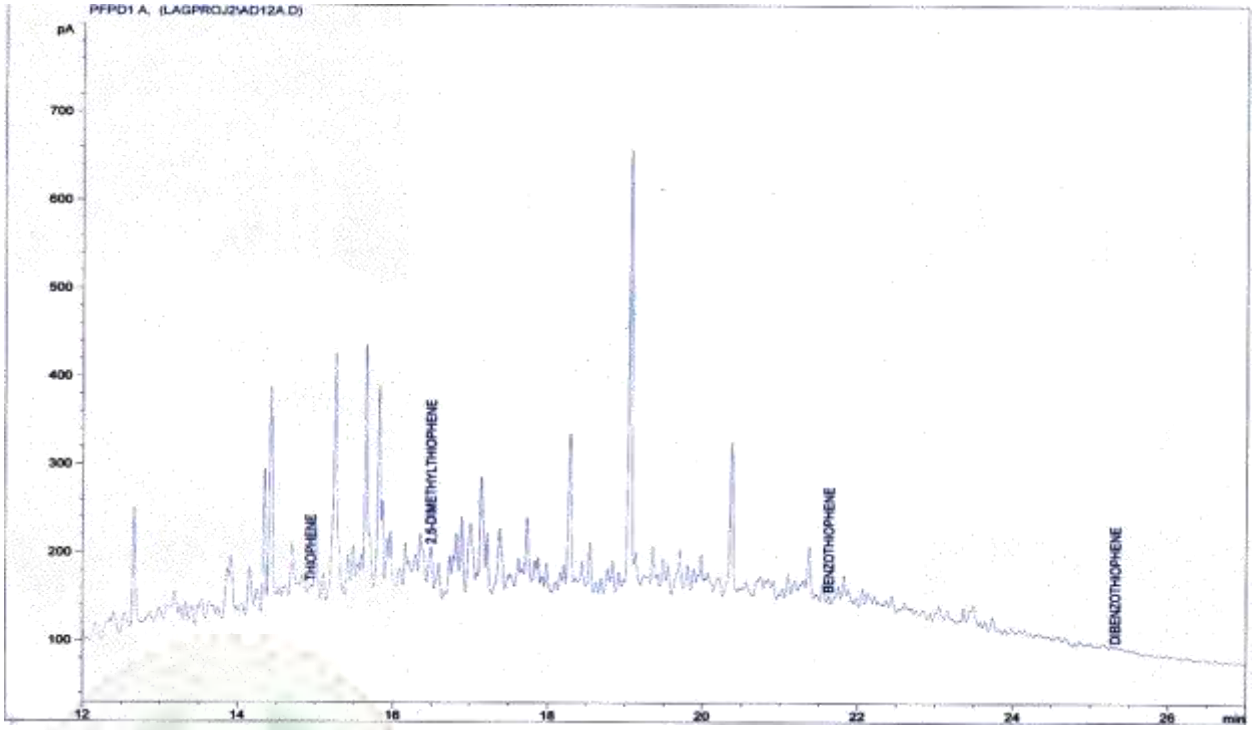

Figure A.9 GC-PFPD Chromatogram for Biodesulfurization of Diesel by *D indolicum* at 12 Hours

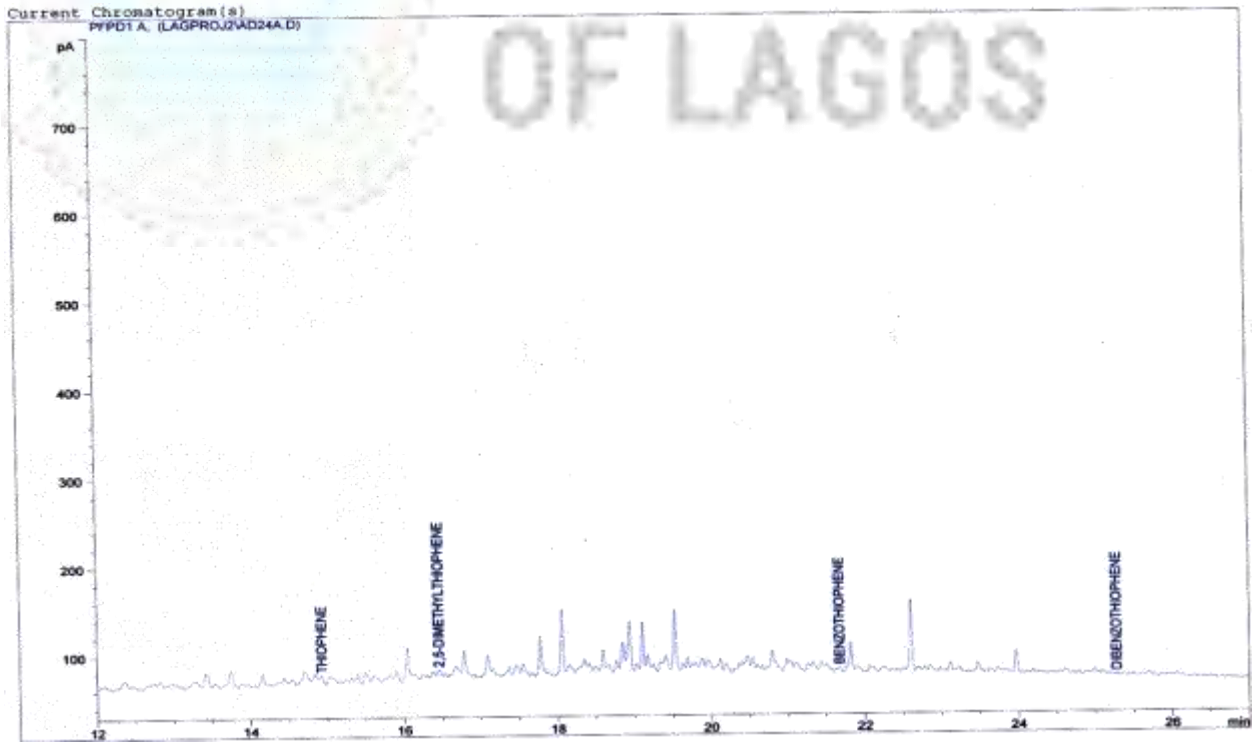

Figure A.10 GC-PFPD Chromatogram for Biodesulfurization of Diesel by *D indolicum* at 24 Hours

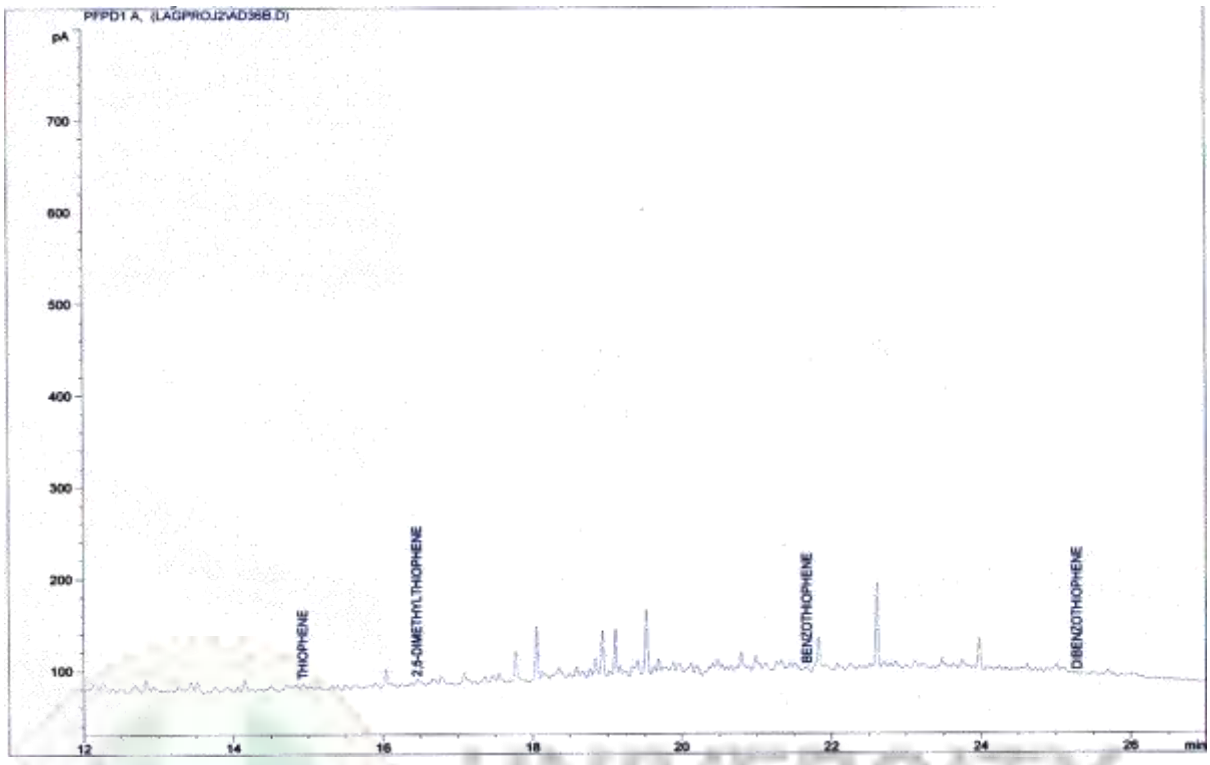

Figure A.11 GC-PFPD Chromatogram for Biodesulfurization of Diesel by *D indolicum* at 36 Hours

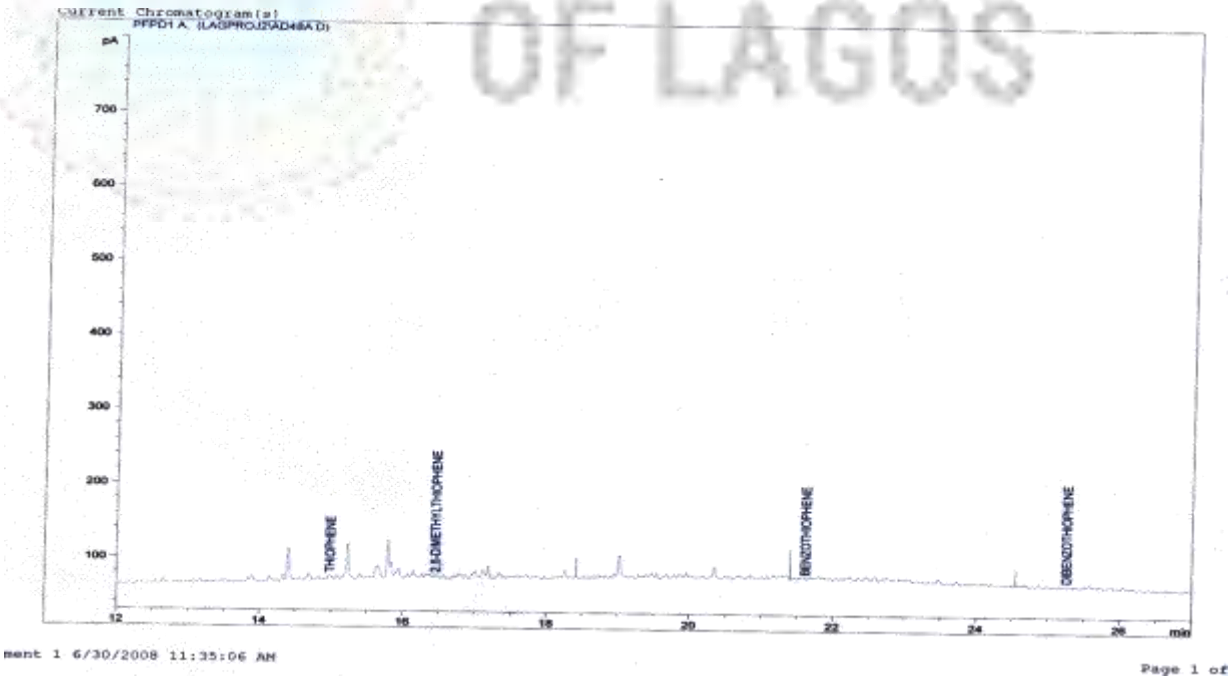

Figure A.12 GC-PFPD Chromatogram for Biodesulfurization of Diesel by *D indolicum* at 48 Hours

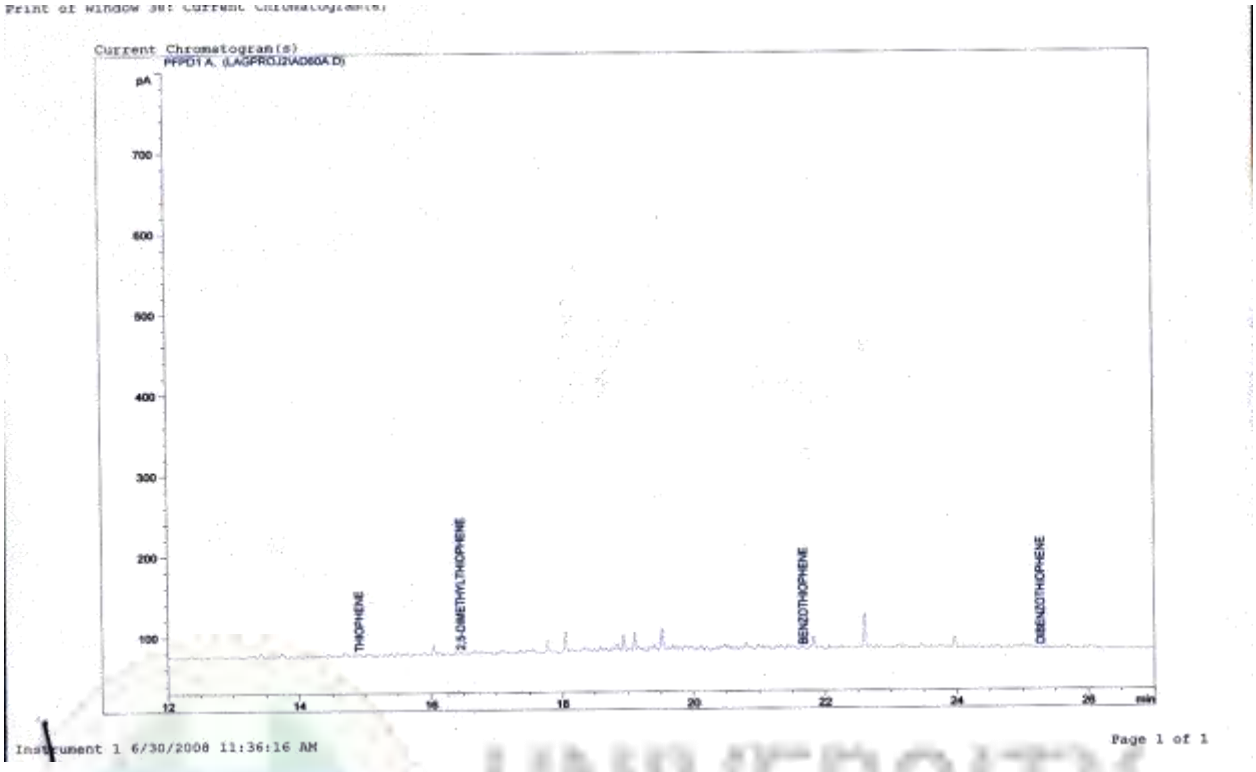

Figure A.13 GC-PFPD Chromatogram for Biodesulfurization of Diesel by *D indolicum* at 60 Hours

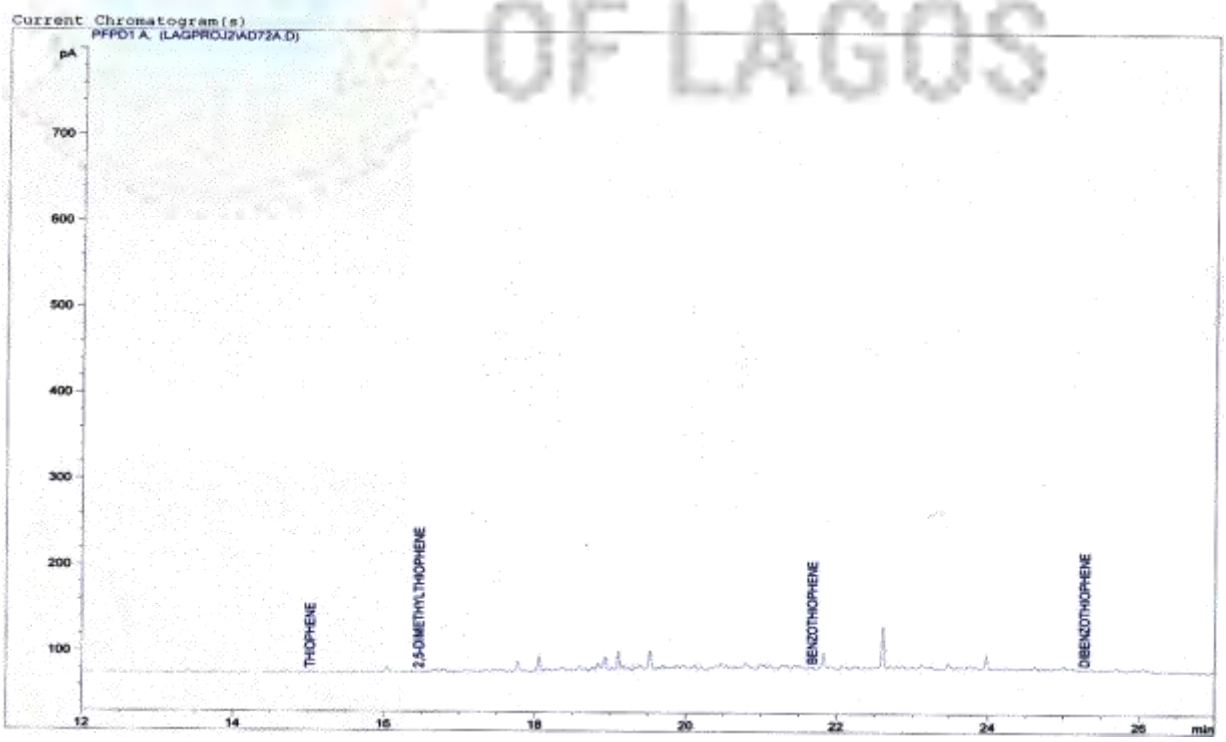

Figure A.14 GC-PFPD Chromatogram for Biodesulfurization of Diesel by *D indolicum* at 72 Hours

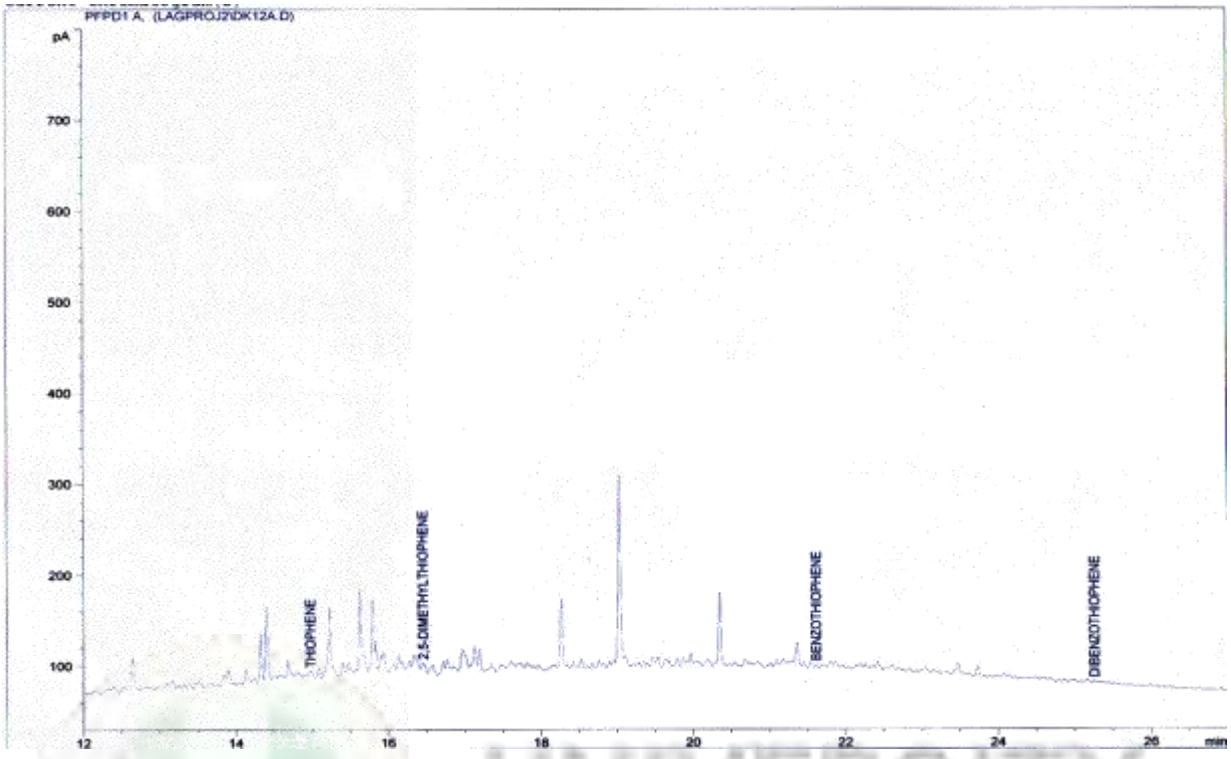

Figure A.15 GC-PFPD Chromatogram for Biodesulfurization of Kerosene by *D anilini* at 12 Hours

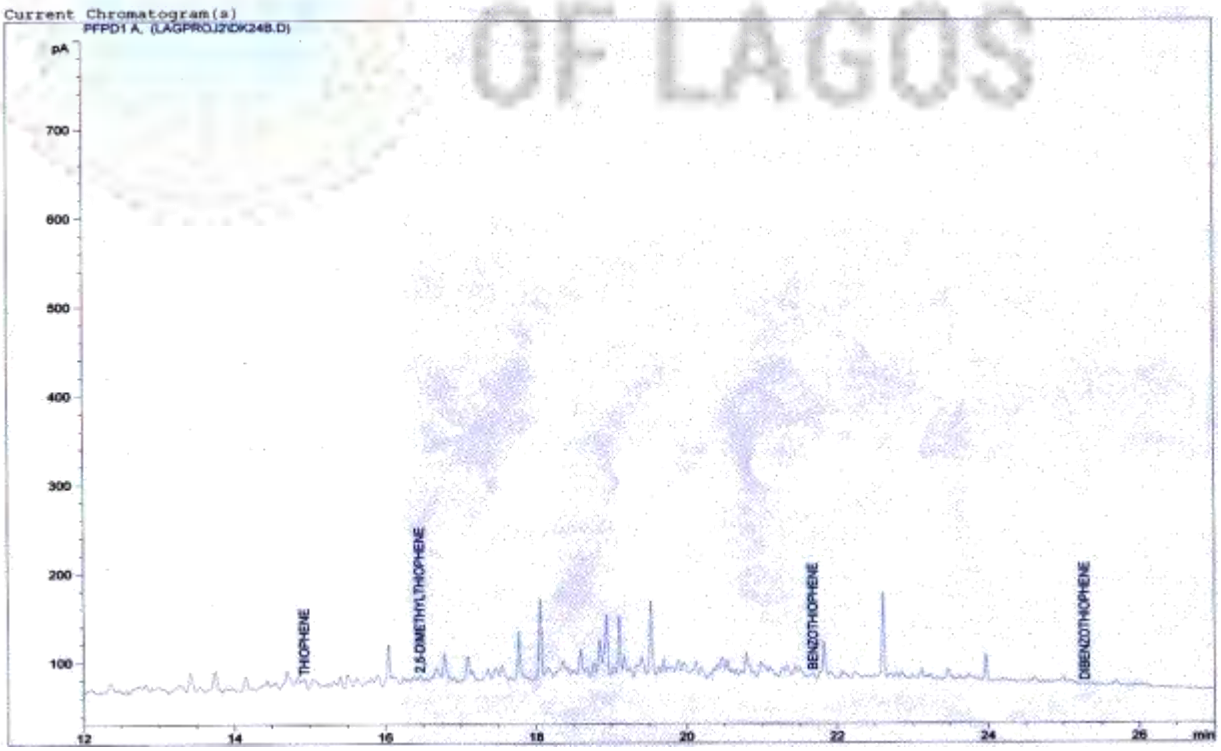

Figure A.16 GC-PFPD Chromatogram for Biodesulfurization of Kerosene by *D anilini* at 24 Hours

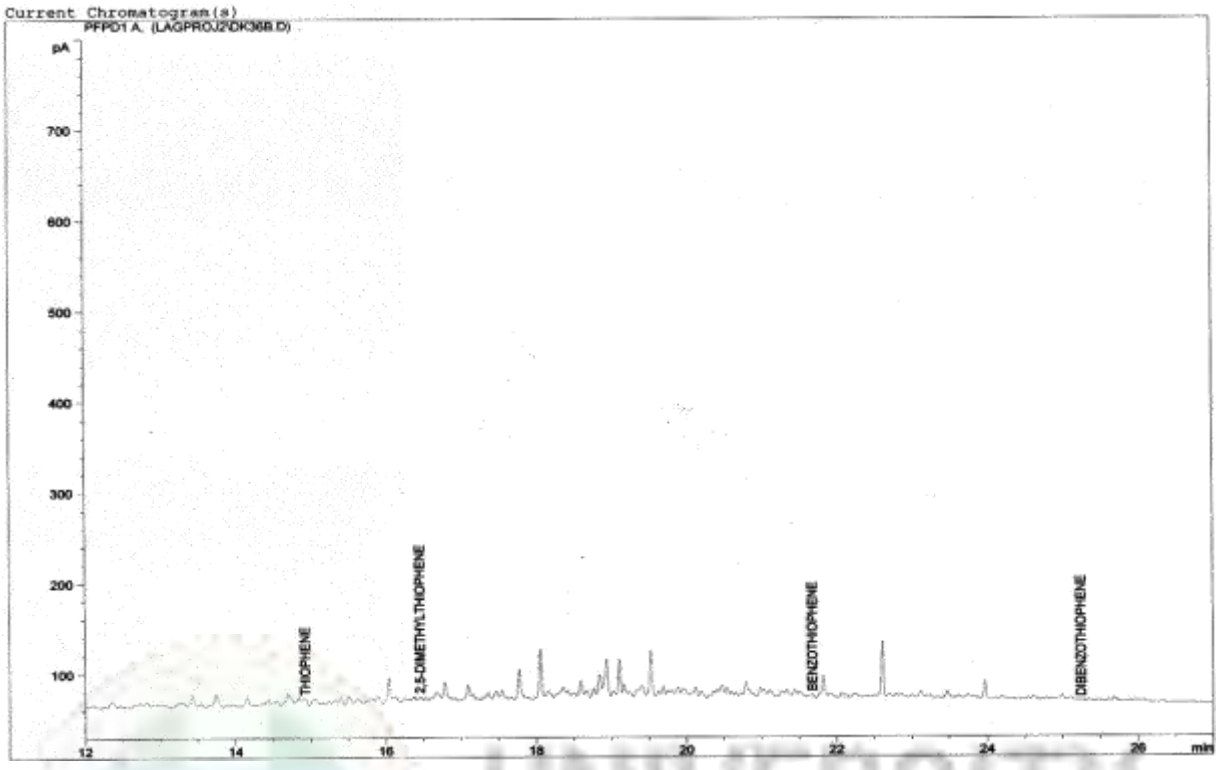

Figure A.17 GC-PFPD Chromatogram for Biodesulfurization of Kerosene by *D anilini* at 36 Hours

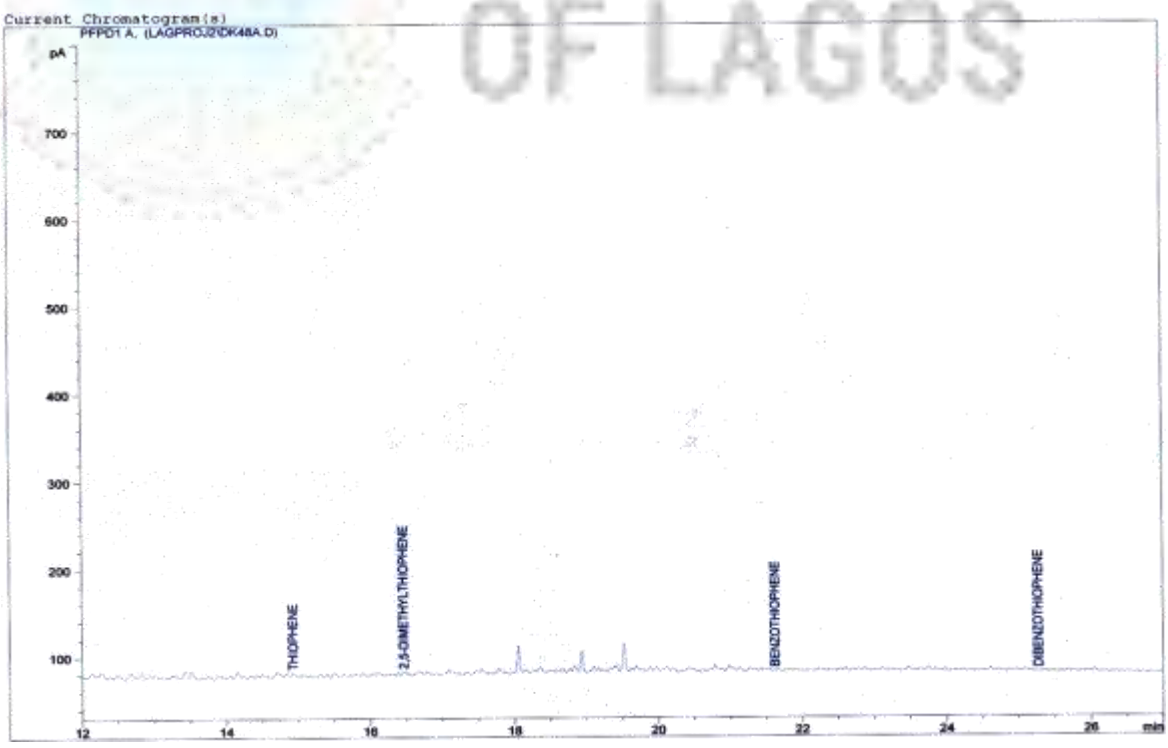

Figure A.18 GC-PFPD Chromatogram for Biodesulfurization of Kerosene by *D anilini* at 48 Hours

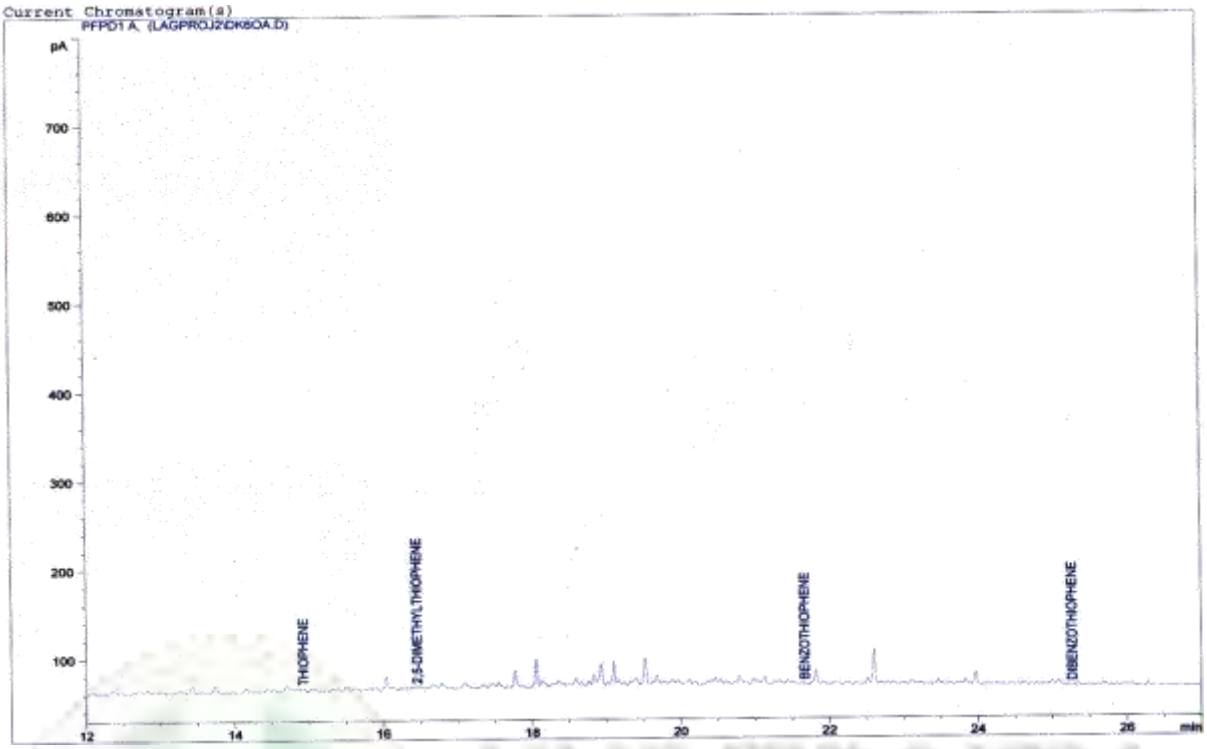

Figure A.19 GC-PFPD Chromatogram for Biodesulfurization of Kerosene by *D anilini* at 60 Hours

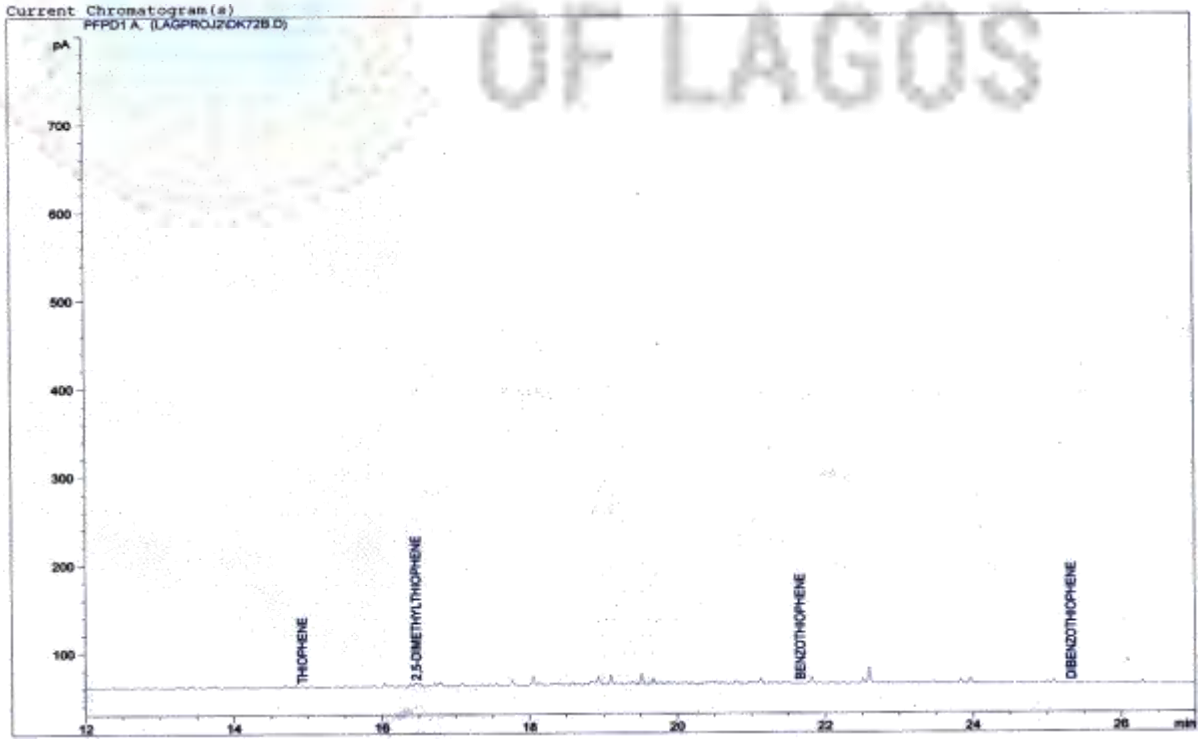

Figure A.20 GC-PFPD Chromatogram for Biodesulfurization of Kerosene by *D anilini* at 72 Hours

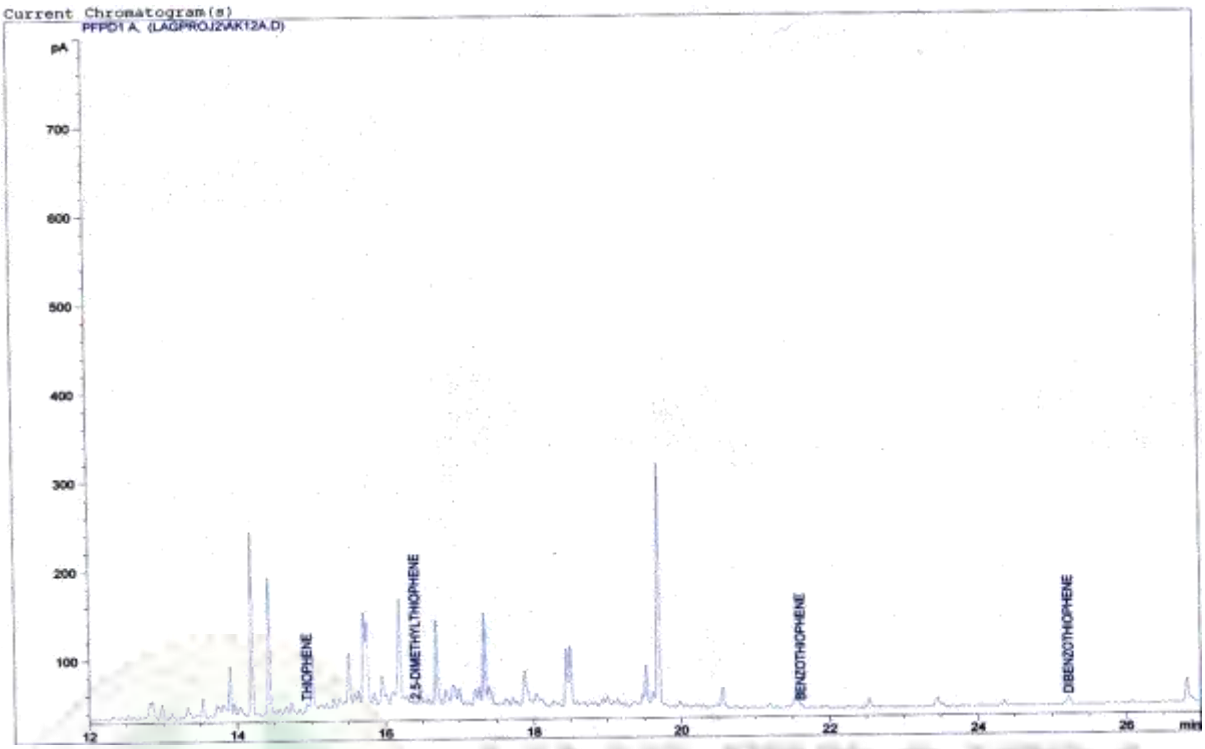

Figure A.21 GC-PFPD Chromatogram for Biodesulfurization of Kerosene by *D indolicum* at 12 Hours

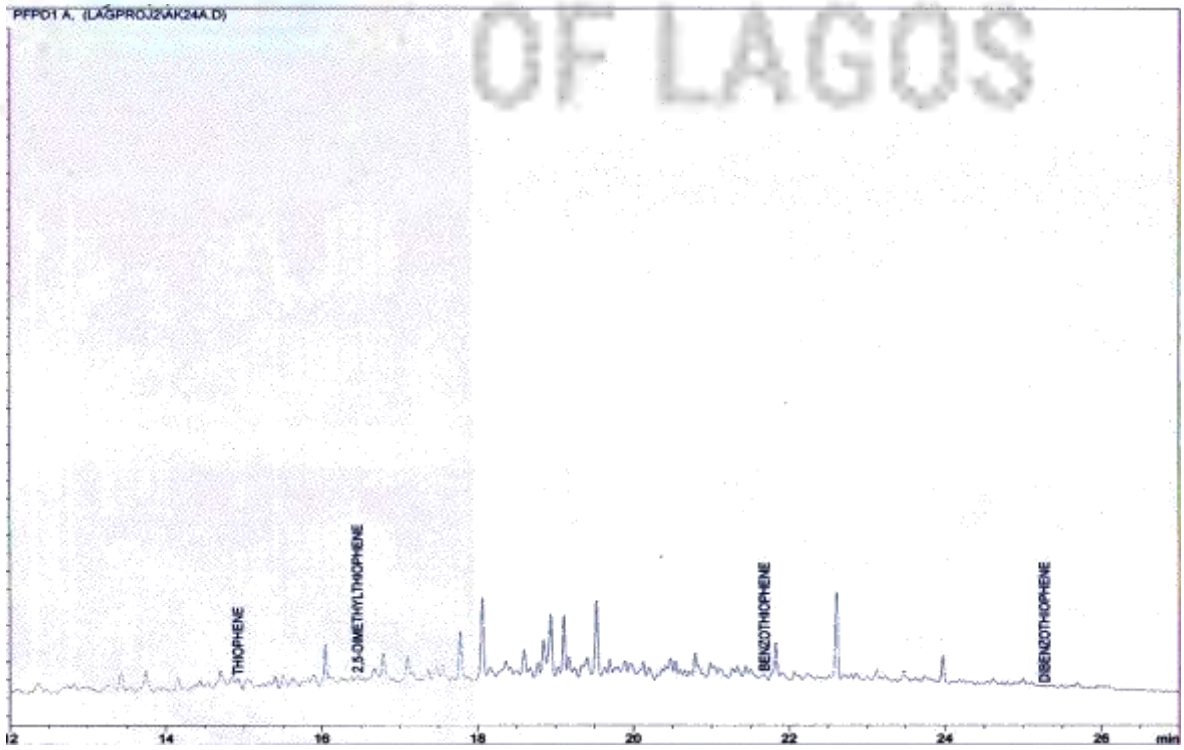

Figure A.22 GC-PFPD Chromatogram for Biodesulfurization of Kerosene by *D indolicum* at 24 Hours

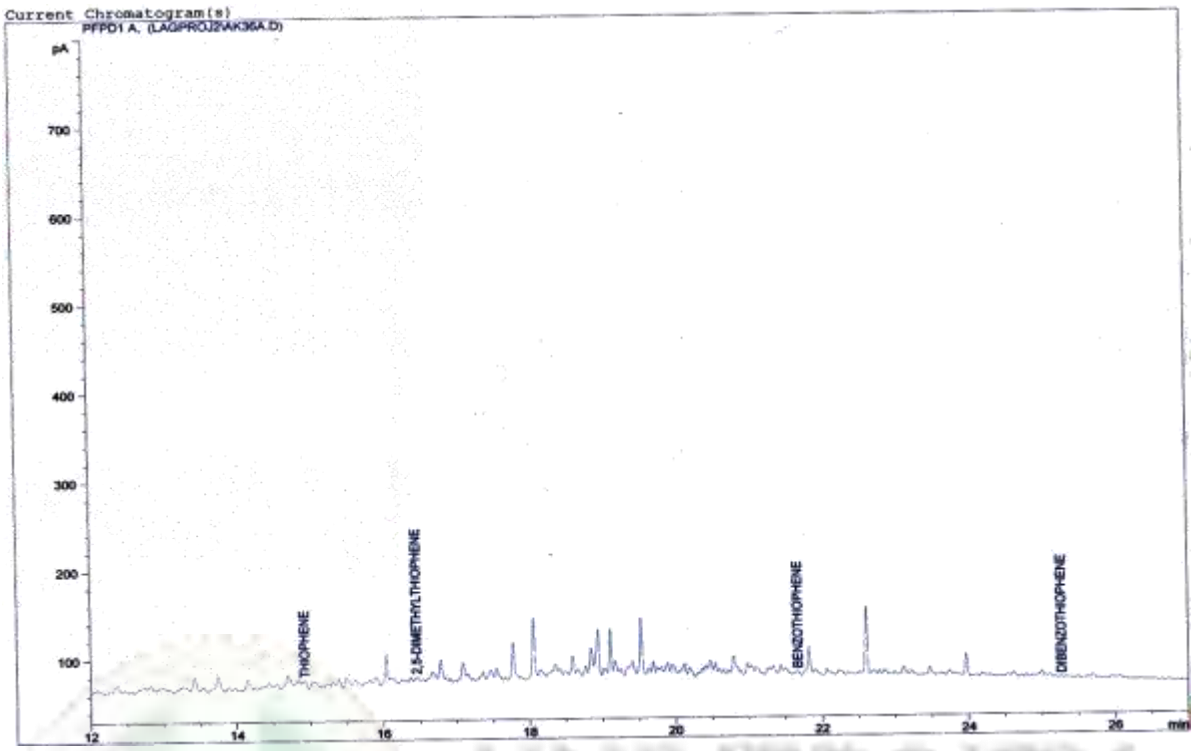

Figure A.23 GC-PFPD Chromatogram for Biodesulfurization of Kerosene by *D indolicum* at 36 Hours

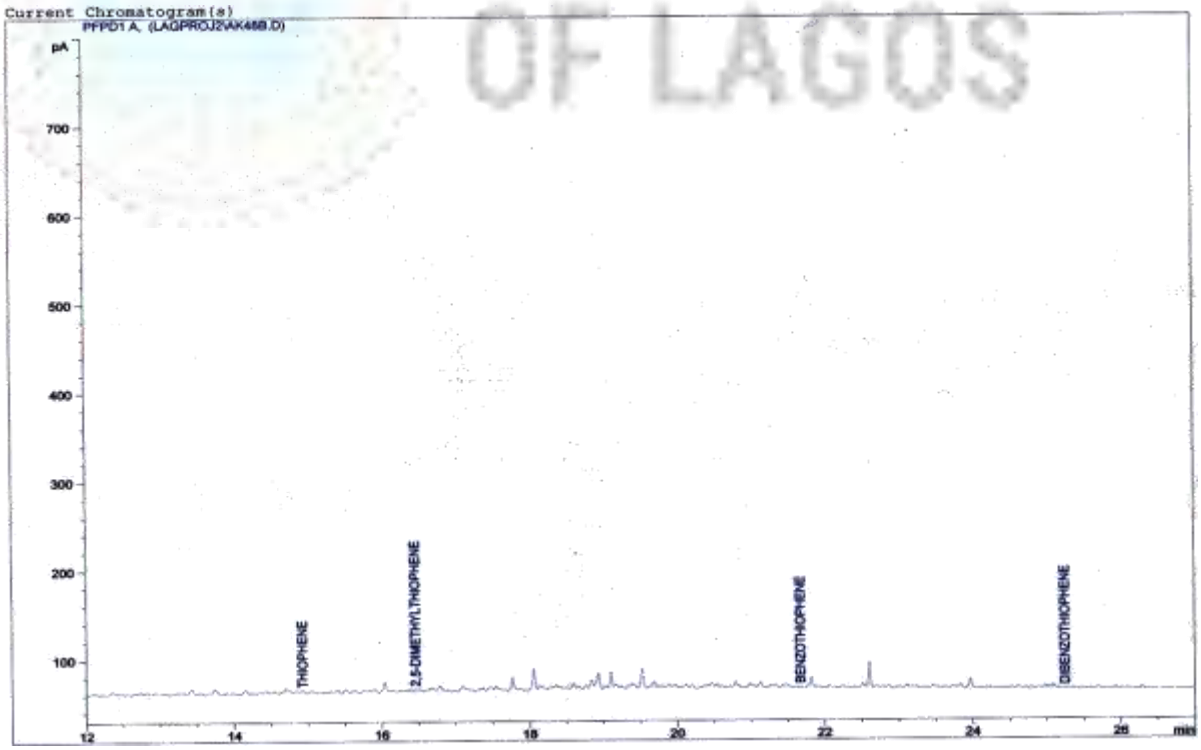

Figure A.24 GC-PFPD Chromatogram for Biodesulfurization of Kerosene by *D indolicum* at 48 Hours

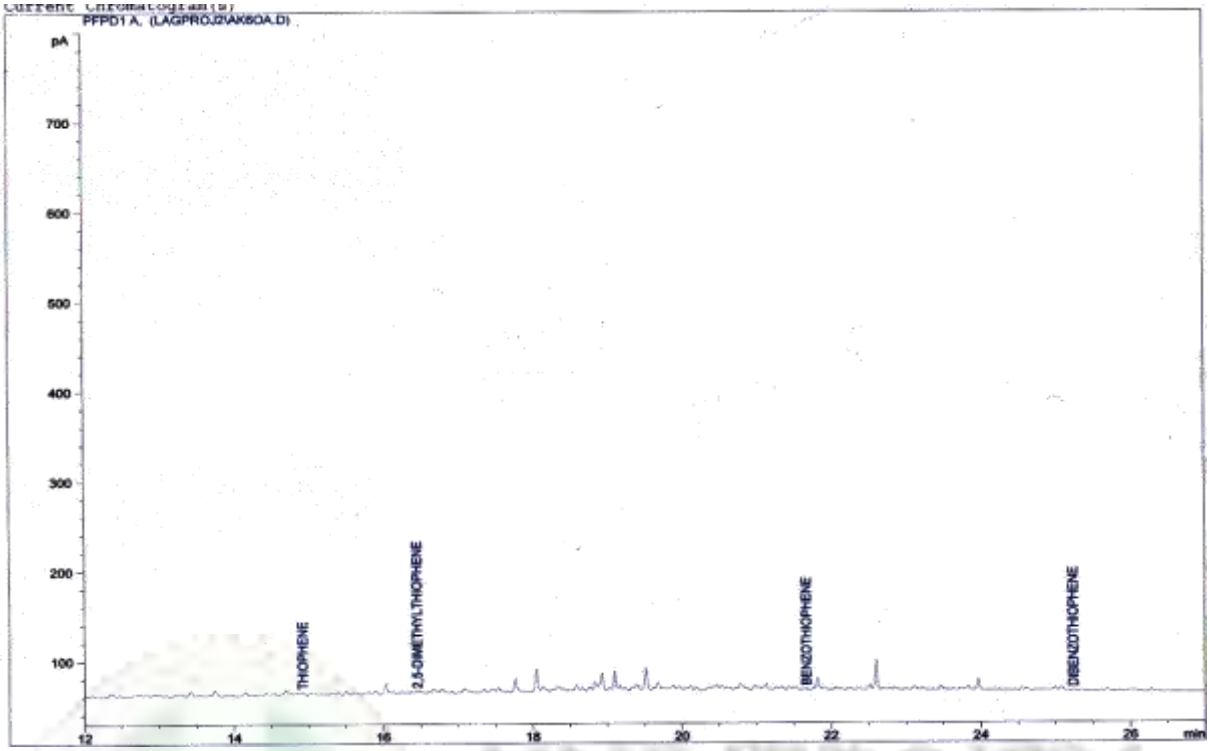

Figure A.25 GC-PFPD Chromatogram for Biodesulfurization of Kerosene by *D indolicum* at 60 Hours

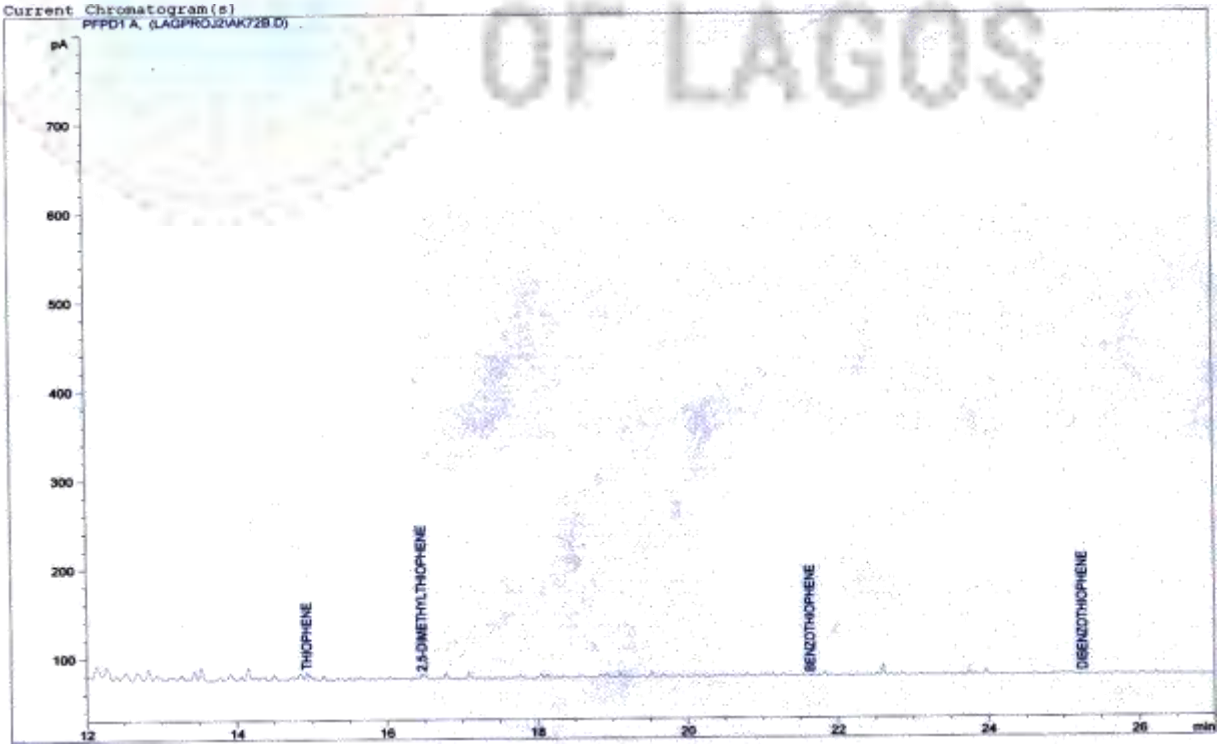

Figure A.26 GC-PFPD Chromatogram for Biodesulfurization of Kerosene by *D indolicum* at 72 Hours

Figure A.27 Benzothiophene GC-PFPD Calibration curve

Figure A.28 Dibenzothiophene GC-PFPD Calibration curve

Figure A.29 Thiophene GC-PFPD Calibration curve

Figure A.30 2, 5 Dimethylthiophene GC-PFPD Calibration Curve

APPENDIX B

TABLES OF THE VARIOUS SUBSTRATES CONCENTRATION – TIME DATA FOR THE TWO MICROORGANISMS

Table B.1: Benzothiophene and Dibenzothiophene Concentration – Time Data during the Biodesulfurization of Diesel by *Desulfobacterium anilini*

t (Hr)	Conc. of Benzothiophene (mg/L)	Conc. of Dibenzothiophene (mg/L)
0	9.006	157.03
12	7.054	125.75
24	5.354	98.937
36	4.357	73.437
48	3.235	53.183
60	2.361	41.965
72	1.681	28.318

Table B.2: Benzothiophene and Dibenzothiophene Concentration – Time Data during the Biodesulfurization of Diesel by *Desulfobacterium indolicum*

t (Hr)	Conc. of Benzothiophene (mg/L)	Conc. of Dibenzothiophene (mg/L)
0	9.006	157.031
12	7.000	123.192
24	5.617	97.947
36	4.343	76.676
48	3.158	55.562
60	2.412	45.4255
72	1.720	31.692

Tables B.3 and B.4 show the biodesulfurization of kerosene by *Desulfobacterium anilini* and D*esulfobacterium indolicum* respectively.

Table B.3: Thiophene and 2 ,5 - Dimethylthiophene Concentration – Time Data during the Biodesulfurization of Kerosene by *Desulfobacterium anilini*

t (Hr)	Conc. of Thiophene (mg/L)	Conc. of 2,5- Dimethylthiophene (mg/L)
0	6.955	41.724
12	5.852	33.301
24	4.652	27.871
36	3.743	22.698
48	2.883	22.010
60	1.861	18.002
72	0.998	11.323

Table B.4: Thiophene and 2 ,5 - Dimethylthiophene Concentration – Time Data during the Biodesulfurization of Kerosene by *Desulfobacterium indolicum*

t (Hr)	Conc. of Thiophene (mg/L)	Conc. of 2,5- Dimethylthiophene (mg/L)
0	6.955	41.724
12	5.859	34.942
24	5.000	30.047
36	3.743	24.590
48	2.619	20.126
60	1.864	16.407
72	1.105	12.353

Appendix C

STATISTICAL SIGNIFICANCE OF EXPERIMENTAL DATA

In this Appendix, the results of the student's t-test statistical analysis performed on the experimental data are shown.

Table C.1: Concentration versus Time Data for Biodesulfurization of Dibenzothiophene by *Desulfobacterium anilini*

t (Hr)	Experimental Data 1(mg/L)	Experimental Data 2 (mg/L)
0	157.031	157.031
12	126.319	125.179
24	99.906	97.968
36	73.166	73.708
48	53.087	53.279
60	41.557	42.373
72	27.779	28.857

t_{exp} = 0.0026, p = 0.9980

Data were found to be significant at the confidence levels of 0.9, 0.95 and 0.99

Table C.2: Concentration versus Time Data for Biodesulfurization of Dibenzothiophene by *Desulfobacterium indolicum*

t (Hr)	Experimental Data 1(mg/L)	Experimental Data 2 (mg/L)
0	157.031	157.031
12	121.903	124.481
24	97.234	98.66
36	79.217	74.135
48	54.305	56.819
60	45.017	45.834
72	31.089	32.295

t_{exp} = 0.0053, p = 0.9840

Data were found to be significant at the confidence levels of 0.9, 0.95 and 0.99

Table C.3: Concentration versus Time Data for Biodesulfurization of Benzothiophene by *Desulfobacterium anilini*

t (Hr)	Experimental Data 1(mg/L)	Experimental Data 2 (mg/L)
0	9.006	9.006
12	7.134	6.974
24	5.21	5.497
36	4.301	4.412
48	3.184	3.286
60	2.395	2.327
72	1.663	1.699

t_{exp} = 0.0272

p = 0.9787

Data were found to be significant at the confidence levels of 0.9, 0.95 and 0.99

Table C.4: Concentration versus Time Data for Biodesulfurization of Benzothiophene by *Desulfobacterium indolicum*

t (Hr)	Experimental Data 1(mg/L)	Experimental Data 2 (mg/L)
0	9.006	9.006
12	7.143	6.856
24	5.836	5.398
36	4.471	4.214
48	3.026	3.289
60	2.482	2.341
72	1.67	1.77

t_{exp} = 0.0774

p = 0.9396

Data were found to be significant at the confidence levels of 0.9, 0.95 and 0.99

Table C.5: Concentration versus Time Data for Biodesulfurization of Thiophene by *Desulfobacterium anilini*

t (Hr)	Experimental Data 1(mg/L)	Experimental Data 2 (mg/L)
0	6.955	6.955
12	5.852	5.852
24	4.751	4.553
36	3.68	3.805
48	2.965	2.801
60	1.811	1.91
72	0.928	1.067

t_{exp} = 0.0338

p = 0.9736

Data were found to be significant at the confidence levels of 0.9, 0.95 and 0.99

Table C.6: Concentration versus Time Data for Biodesulfurization of Thiophene by *Desulfobacterium indolicum*

t (Hr)	Experimental Data 1(mg/L)	Experimental Data 2 (mg/L)
0	6.955	6.955
12	5.835	5.882
24	4.725	5.275
36	3.684	3.802
48	2.606	2.632
60	1.878	1.850
72	1.173	1.036

t_{exp} = 0.0712

p = 0.9843

Data were found to be significant at the confidence levels of 0.9, 0.95 and 0.99

Table C.7: Concentration versus Time Data for Biodesulfurization of 2, 5 - Dimethylthiophene by *Desulfobacterium anilini*

t (Hr)	Experimental Data 1(mg/L)	Experimental Data 2 (mg/L)
0	41.724	41.724
12	34.122	32.479
24	28.194	27.548
36	22.483	22.913
48	21.799	22.22
60	17.863	18.14
72	11.376	11.27

$t_{exp} = 0.0337$

$p = 0.9737$

Data were found to be significant at the confidence levels of 0.9, 0.95 and 0.99

Table C.8: Concentration versus Time Data for Biodesulfurization of 2, 5 - Dimethylthiophene by *Desulfobacterium indolicum*

t (Hr)	Experimental Data 1(mg/L)	Experimental Data 2 (mg/L)
0	41.724	41.724
12	35.424	34.459
24	30.335	29.759
36	24.389	24.791
48	19.776	20.475
60	16.587	16.226
72	12.346	12.36

$t_{exp} = 0.0201$

$p = 0.9843$

Data were found to be significant at the confidence levels of 0.9, 0.95 and 0.99

APPENDIX D

THE TABLES SHOW THE DATA USED FOR THE PLOTTING OF FIGURES 4.1 TO 4.24

Table D.1: Table Showing Experimental and Simulated Kinetic Models of Biodesulfurization of DBT in Diesel By *Desulfobacterium anilini*

Time (Hr)	Experimental D.Data (mg/L)	Simulated Data of Kinetic with Mass Transfer Model (mg/L)	Simulated Data of Kinetic without Mass Transfer Model (mg/L)
0	0	0	0
12	0.199	0.215	0.166
24	0.370	0.375	0.336
36	0.532	0.488	0.456
48	0.661	0.629	0.606
60	0.733	0.716	0.698
72	0.820	0.802	0.790

Table D.2: Table Showing Experimental and Simulated Kinetic Models of Biodesulfurization of Benzothiophene in Diesel by *Desulfobacterium anilini*

Time (Hr)	Experimental Data (mg/L)	Simulated Data of Kinetic with Mass Transfer Model (mg/L)	Simulated Data of Kinetic without Mass Transfer Model (mg/L)
0	0	0	0
12	0.217	0.235	0.208
24	0.406	0.384	0.362
36	0.516	0.513	0.496
48	0.641	0.608	0.594
60	0.738	0.714	0.704
72	0.813	0.792	0.785

Table D.3: Table Showing Experimental and Simulated Kinetic Models of Biodesulfurization of 2, 5 – Dimethylthiophene in Kerosene by *Desulfobacterium anilini*

Time (Hr)	Experimental Data (mg/L)	Simulated Data of Kinetic with Mass Transfer Model (mg/L)	Simulated Data of Kinetic without Mass Transfer Model (mg/L)
0	0	0	0
12	0.202	0.174	0.130
24	0.332	0.311	0.275
36	0.456	0.417	0.386
48	0.472	0.498	0.471
60	0.569	0.561	0.538
72	0.729	0.659	0.641

Table D.4: Table Showing Experimental and Simulated Kinetic Models of Biodesulfurization of Thiophene in Kerosene by *Desulfobacterium anilini*

Time (Hr)	Experimental Data (mg/L)	Simulated Data of Kinetic with Mass Transfer Model (mg/L)	Simulated Data of Kinetic without Mass Transfer Model (mg/L)
0	0	0	0
12	0.159	0.155	0.096
24	0.331	0.307	0.258
36	0.463	0.452	0.413
48	0.585	0.586	0.558
60	0.732	0.704	0.684
72	0.857	0.824	0.812

Table D.5: Table Showing Experimental and Simulated Kinetic Models of Biodesulfurization of DBT in Diesel By *Desulfobacterium indolicum*

Time (Hr)	Experimental Data (mg/L)	Simulated Data of Kinetic with Mass Transfer Model (mg/L)	Simulated Data of Kinetic without Mass Transfer Model (mg/L)
0	0	0	0
12	0.215	0.230	0.185
24	0.376	0.365	0.324
36	0.512	0.524	0.494
48	0.646	0.647	0.625
60	0.711	0.722	0.704
72	0.798	0.820	0.809

Table D.6: Table Showing Experimental and Simulated Kinetic Models of Biodesulfurization of Benzothiophene in Diesel by *Desulfobacterium indolicum*

Time (Hr)	Experimental Data (mg/L)	Simulated Data of Kinetic with Mass Transfer Model (mg/L)	Simulated Data of Kinetic without Mass Transfer Model (mg/L)
0	0	0	0
12	0.223	0.230	0.202
24	0.376	0.381	0.359
36	0.518	0.484	0.465
48	0.649	0.607	0.593
60	0.732	0.675	0.663
72	0.809	0.734	0.725

Table D.7: Table Showing Experimental and Simulated Kinetic Models of Biodesulfurization of 2, 5 – Dimethylthiophene in Kerosene by *Desulfobacterium indolicum*

Time (Hr)	Experimental Data (mg/L)	Simulated Data of Kinetic with Mass Transfer Model (mg/L)	Simulated Data of Kinetic without Mass Transfer Model (mg/L)
0	0	0	0
12	0.163	0.160	0.115
24	0.280	0.288	0.250
36	0.411	0.389	0.357
48	0.518	0.493	0.466
60	0.607	0.556	0.532
72	0.704	0.618	0.598

Table D.8: Table Showing Experimental and Simulated Kinetic Models of Biodesulfurization of Thiophene in Kerosene by *Desulfobacterium indolicum*

Time (Hr)	Experimental Data (mg/L)	Simulated Data of Kinetic with Mass Transfer Model (mg/L)	Simulated Data of Kinetic without Mass Transfer Model (mg/L)
0	0	0	0
12	0.158	0.153	0.094
24	0.281	0.302	0.253
36	0.462	0.445	0.407
48	0.623	0.621	0.595
60	0.732	0.694	0.673
72	0.841	0.784	0.769

APPENDIX E

ESTIMATING PARTITION (DISTRIBUTION) COEFFICIENT

The molar volume of diesel and kerosene are estimated as their respective volumes at their boiling points.

The boiling point of kerosene ranges from 120 – 170^{0}C

That of diesel ranges from 190 – 250^{0}C depending on the composition. For the purpose of this estimation, the midpoints of the ranges were chosen and thus estimated thus:

$$V_{f,kero} = \frac{RT}{P} = \frac{8.314 \cdot (273 + 145)}{101325} = 0.034 \text{ m}^3$$

$$V_{f,diesel} = \frac{RT}{P} = \frac{8.314 \cdot (273 + 220)}{101325} = 0.04 \text{ m}^3$$

The vapor pressures of the components of the *Fuel*s are shown below

Components	Vapor pressure at ambient temp (kPa)	
Thiophene	43.3	
2, 5-dimethylthiophene	6.98	
Benzothiophene	0.76	
Dibenzothiophene	0.35	(Pe´rez-Pellitero *et al*, 2007)

The air-water linear solvation energy relationships (LSERs) coefficients are as follows

	c	**r**	**s**	**a**	**b**	**v**	
water-air	-0.99	0.58	2.55	3.81	4.84	-0.90	(Abraham *et al*, 1994)

The LSER Solute Parameters

Solute	R_2	Π_2^H	α_2^H	β_2^H	Vx
Thiophene	0.687	0.56	0.00	0.15	0.641
2, 5-dimethylthiophene	1.031	0.84	0.00	0.225	0.962
Benzothiophene	1.323	0.88	0.00	0.20	1.010
Dibenzothiophene	1.588	0.956	0.00	0.28	1.312

Log $K_{Thiop,,fw}$ =

$$\log\left(8.314\frac{303}{0.034433}\right) - (-0.99 + 0.58\,0.687 + 2.55\,0.56 + 4.84\,0.15 + -0.9\,0.641) = 0.248$$

$K_{Thiop,,fw}$ = $\exp(0.248) = 1.281$

Log $K_{2,5\text{-dimeththiop},,fw}$ =

$$\log\left(8.314\frac{303}{0.034698}\right) - (-0.99 + 0.58\,1.031 + 2.55\,0.84 + 4.84\,0.225 + -0.9\,0.962) = 0.053$$

$K_{2,5\text{-dimeththiop},,fw}$ = $\exp(0.053) = 1.054$

Log $K_{Benzthiop,,fw}$ = $\log\left(8.314\frac{303}{0.0476}\right) - (-0.99 + 0.58\,1.323 + 2.55\,0.88 + 4.84\,0.20 + -0.9\,1.010) = 0.838$

$K_{Benzthiop,,fw}$ = $\exp(0.838) = 2.312$

Log $K_{Dibenzthiop,,fw}$ = $\log\left(8.314\frac{303}{0.0435}\right) - (-0.99 + 0.58\,1.588 + 2.55\,0.956 + 4.84\,0.28 + -0.9\,1.312) = 0.712$

$K_{Dibenzthiop,,fw}$ = $\exp(0.712) = 2.038$

APPENDIX F

FINITE DIFFERENCE METHOD SUB ROUTINE USED TO SOLVE THE DEVELOPED KINETIC MODEL OF BIODESULFURIZATION

$$\frac{dC}{dt} = \frac{kC}{K_M + C}$$

$$\frac{C_i^{n+1} - C_i^n}{\Delta t} = \frac{kC_i^n}{K_M + C_i^n}$$

$$C_i^{n+1} - C_i^n = \frac{kC_i^n \Delta t}{K_M + C_i^n}$$

$$C_i^{n+1} = C_i^n + \frac{kC_i^n \Delta t}{K_M + C_i^n}$$

FINITE DIFFERENCE NUMERICAL METHOD FOR SOLVING THE DEVELOPED KINETIC MODEL OF BIODESULFURIZATION WITH MASS TRANSFER FACTOR

$$\frac{dC}{dt} = \frac{kC}{(K_M + C)(1+K)}$$

$$\frac{C_i^{n+1} - C_i^n}{\Delta t} = \frac{kC_i^n}{(K_M + C_i^n)(1+K)}$$

$$C_i^{n+1} - C_i^n = \frac{kC_i^n \Delta t}{(K_M + C_i^n)(1+K)}$$

$$C_i^{n+1} = C_i^n + \frac{kC_i^n \Delta t}{(K_M + C_i^n)(1+K)}$$

FILE ABDD 1 FOR

```
C       BIODESULFURIZATION KINETICS OF THIOPHENE BY ANILINI
REAL C(7),DT(7),KA,KM
DATA KA,KM/0.104,0.548/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
36.0,48.0,60.0,72.0/
OPEN(6,FILE='ABD1.RES')
C DEFINING INITIAL CONDITION
C(1)=6.95523
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```


RESULT OF THE KINETIC MODEL
FILE ABD 1 RES

DT(K)	C(K)
12.00	6.2853
24.00	5.1583
36.00	4.0839
48.00	3.0767
60.00	2.2004
72.00	1.3089

FILE ABDD 2 FOR

```
C BIODESULFURIZATION KINETICS OF 2,5 DIMETHYLTHIOPHENE BY ANILINI
REAL C(7),DT(7),KA,KM
DATA KA,KM/1.426,51.700/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
36.0,48.0,60.0,72.0/
OPEN(6,FILE='ABD2.RES')
C DEFINING INITIAL CONDITION
C(1)=6.95523
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```

RESULT OF THE KINETIC MODEL
FILE ABD 2 RES

DT(K)	C(K)
12.00	36.2992
24.00	30.2553
36.00	25.6104
48.00	22.0523
60.00	19.2863
72.00	14.9887

FILE ABDD 3 FOR

```
C       BIODESULFURIZATION KINETICS OF THIOPHENE BY INDOLICUM
REAL C(7),DT(7),KA,KM
DATA KA,KM/0.103,0.575/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
36.0,48.0,60.0,72.0/
OPEN(6,FILE='ABD3.RES')
C DEFINING INITIAL CONDITION
C(1)=6.95523
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```


RESULT OF THE KINETIC MODEL
FILE ABD 3 RES

DT(K)	C(K)
12.00	6.3003
24.00	5.1948
36.00	4.1267
48.00	2.8192
60.00	2.2746
72.00	1.6040

FILE ABDD 4 FOR

```
C       BIODESULFURIZATION KINETICS OF 2,5-DIMETHYLTHIOPHENE BY
INDOLICUM
REAL C(7),DT(7),KA,KM
DATA KA,KM/1.350,54.700/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
36.0,48.0,60.0,72.0/
OPEN(6,FILE='ABD4.RES')
C DEFINING INITIAL CONDITION
C(1)=6.95523
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```


RESULT OF THE KINETIC MODEL
FILE ABD 4 RES

DT(K)	C(K)
12.00	36.9261
24.00	31.2793
36.00	26.8310
48.00	22.2893
60.00	19.5230
72.00	16.7657

FILE ABDD 5 FOR

```
C       BIODESULFURIZATION KINETICS OF BENZOTHIOPHENE BY ANILINI
REAL C(7),DT(7),KA,KM
DATA KA,KM/0.572,18.050/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
36.0,48.0,60.0,72.0/
OPEN(6,FILE='ABD5.RES')
C DEFINING INITIAL CONDITION
C(1)=9.00615
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```


RESULT OF THE KINETIC MODEL
FILE ABD 5 RES

DT(K)	C(K)
12.00	7.1369
24.00	5.7543
36.00	4.5403
48.00	3.6538
60.00	2.6663
72.00	1.9378

FILE ABDD 6 FOR

```
C       BIODESULFURIZATION KINETICS OF DIBENZOTHIOPHENE BY ANILINI
REAL C(7),DT(7),KA,KM
DATA KA,KM/6.118,182.278/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
36.0,48.0,60.0,72.0/
OPEN(6,FILE='ABD6.RES')
C DEFINING INITIAL CONDITION
C(1)=157.0311
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```


RESULT OF THE KINETIC MODEL
FILE ABD 6 RES

DT(K)	C(K)
12.00	131.0308
24.00	104.3456
36.00	85.4824
48.00	61.9112
60.00	47.4493
72.00	32.9814

FILE ABDD 7 FOR

```
C       BIODESULFURIZATION KINETICS OF BENZOTHIOPHENE BY INDOLICUM
REAL C(7),DT(7),KA,KM
DATA KA,KM/0.540,19.837/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
36.0,48.0,60.0,72.0/
OPEN(6,FILE='ABD7.RES')
C DEFINING INITIAL CONDITION
C(1)=9.00615
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```


RESULT OF THE KINETIC MODEL
FILE ABD 7 RES

DT(K)	C(K)
12.00	7.1883
24.00	5.7743
36.00	4.8143
48.00	3.6662
60.00	3.0333
72.00	2.4784

FILE ABDD 8 FOR

```
C       BIODESULFURIZATION KINETICS OF DIBENZOTHIOPHENE BY INDOLICUM
REAL C(7),DT(7),KA,KM
DATA KA,KM/5.992,192.782/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
36.0,48.0,60.0,72.0/
OPEN(6,FILE='ABD8.RES')
C DEFINING INITIAL CONDITION
C(1)=157.0311
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```

RESULT OF THE KINETIC MODEL
FILE ABD 8 RES

DT(K)	C(K)
12.00	128.0283
24.00	106.0776
36.00	79.4824
48.00	58.8772
60.00	46.4793
72.00	30.0144

FILE ABDD 9 FOR

```
C       BIODESULFURIZATION OF BENZOTHIOPHENE BY ANILINI WITH MASS
TRANS
REAL C(7),DT(7),KA,KM,KD
DATA KA,KD,KM/0.572,2.312,18.05/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
36.0,48.0,60.0,72.0/
OPEN(6,FILE='ABD9.RES')
C DEFINING INITIAL CONDITION
C(1)=9.00615
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(1.0/(1.0+KD))*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```


RESULT OF THE KINETIC MODEL
FILE ABD 9 RES

DT(K)	C(K)
12.00	6.8894
24.00	5.5504
36.00	4.3822
48.00	3.5273
60.00	2.5734
72.00	1.8711

FILE ABDD 10 FOR

```
C       BIODESULFURIZATION OF DIBENZOTHIOPHENE BY ANILINI WITH MASS
TRANS
REAL C(7),DT(7),KA,KD,KM
DATA KA,KD,KM/6.118,2.038,182.278/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
36.0,48.0,60.0,72.0/
OPEN(6,FILE='ABD10.RES')
C DEFINING INITIAL CONDITION
C(1)=157.0311
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(1.0/(1.0+KD))*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```


RESULT OF THE KINETIC MODEL
FILE ABD 10 RES

DT(K)	C(K)
12.00	123.2651
24.00	98.1621
36.00	80.4161
48.00	58.2421
60.00	44.6378
72.00	31.0262

FILE ABDD 11 FOR

```
C       BIODESULFURIZATION OF BENZOTHIOPHENE BY INDOLICUM WITH MASS
TRANS
REAL C(7),DT(7),KA,KM,KD
DATA KA,KD,KM/0.572, 2.312,19.837/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
136.0,48.0,59.3,72.7/
OPEN(6,FILE='ABD111.RES')
C DEFINING INITIAL CONDITION
C(1)=9.00615
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(1.0/(1.0+KD))*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```

RESULT OF THE KINETIC MODEL
FILE ABD 11 RES

DT(K)	C(K)
12.00	6.9378
24.00	5.5728
36.00	4.6469
48.00	3.5392
60.00	2.9283
72.00	2.3918

FILE ABDD 12 FOR

```
C       BIODESULFURIZATION OF DIBENZOTHIOPHENE BY INDOLICUM WITH
MASS TRANS
REAL C(7),DT(7),KA,KD,KM
DATA KA,KD,KM/5.992,2.038,192.782/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
36.0,48.0,60.0,72.0/
OPEN(6,FILE='ABD 12.RES')
C DEFINING INITIAL CONDITION
C(1)=157.0311
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(1.0/(1.0+KD))*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```

RESULT OF THE KINETIC MODEL
FILE ABD 12 RES

DT(K)	C(K)
12.00	120.9439
24.00	99.7911
36.00	74.7709
48.00	55.3877
60.00	43.7242
72.00	28.2354

FILE ABDD 13 FOR

```
C       BIODESULFURIZATION OF THIOPHENE BY ANILINI WITH MASS TRANS
REAL C(7),DT(7),KA,KD,KM
DATA KA,KD,KM/0.104,1.218,0.548/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
36.0,48.0,60.0,72.0/
OPEN(6,FILE='ABD13.RES')
C DEFINING INITIAL CONDITION
C(1)=6.95523
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(1.0/(1.0+KD))*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```


RESULT OF THE KINETIC MODEL
FILE ABD 13 RES

DT(K)	C(K)
12.00	5.8741
24.00	4.8216
36.00	3.8133
48.00	2.8760
60.00	2.0563
72.00	1.2229

FILE ABDD 14 FOR

```
C       BIODESULFURIZATION OF DIMETHYLTHIOPHNE BY ANILINI WITH MASS
TRANS
REAL C(7),DT(7),KA,KD,KM
DATA KA,KD,KM/1.426,1.054,51.7/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
36.0,48.0,60.0,72.0/
OPEN(6,FILE='ABD14.RES')
C DEFINING INITIAL CONDITION
C(1)=41.72435
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(1.0/(1.0+KD))*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```


RESULT OF THE KINETIC MODEL
FILE ABD 14 RES

DT(K)	C(K)
12.00	34.4717
24.00	28.7318
36.00	24.3214
48.00	20.9427
60.00	18.3154
72.00	14.2352

FILE ABDD 15 FOR

```
C       BIODESULFURIZATION OF THIOPHENE BY INDOLICUM WITH MASS TRANS
REAL C(7),DT(7),KA,KD,KM
DATA KA,KD,KM/0.103,1.281,0.575/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
36.0,48.0,60.0,72.0/
OPEN(6,FILE='ABD 15.RES')
C DEFINING INITIAL CONDITION
C(1)=6.95523
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(1.0/(1.0+KD))*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```

RESULT OF THE KINETIC MODEL
FILE ABD 15 RES

DT(K)	C(K)
12.00	5.8883
24.00	4.8550
36.00	3.8571
48.00	2.6348
60.00	2.1259
72.00	1.4993

FILE ABDD 16 FOR

```
C       BIODESULFURIZATION OF DIMETHYLTHIOPHNE BY INDOLICUM WITH
MASS TRANS
REAL C(7),DT(7),KA,KD,KM
DATA KA,KD,KM/1.35,1.054,54.7/
DATA DT(1),DT(2),DT(3),DT(4),DT(5),DT(6),DT(7)/0.0,12.0,24.0,
136.0,48.0,60.0,73.5/
OPEN(6,FILE='ABD 16.RES')
C DEFINING INITIAL CONDITION
C(1)=41.72436
WRITE(6,30)'DT(K)','C(K)'
30  FORMAT(3X,A5,6X,A5)
DO 10 K=2,7
C(K)=C(1)-DT(K)*(1.0/(1.0+KD))*(KA*C(K-1)/(KM+C(K-1)))
WRITE(6,32) DT(K),C(K)
32  FORMAT(2X,F5.2,3X,F8.4)
10  CONTINUE
END
```


FILE ABD 16 RES

```
DT(K)      C(K)
12.00    35.0666
24.00    29.7045
36.00    25.4814
48.00    22.1673
60.00    18.5401
72.00    15.9221
```