BIODEGRADATION OF INDUSTRIAL RAW MATERIALS AND THEIR PRODUCTS

by

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of the University of the Witwatersrand
in fulfilment of the requirements for the degree
of Master of Science

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I would like to dedicate this Dissertation:

To my parents, Anand & Shirley Ramsuran,

To my sisters, Praveena & Raksha &

The memory of my grandfather

DECLARATION

I declare that this Dissertation is my own unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

_____

Nisha Anand Ramsuran

_____day of _____2011

This dissertation is submitted after examination and approval by the following supervisor:

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ABSTRACT

Biodegradation and green chemistry technologies have become increasing common demands in the industrial sector. The fate and potential impact of industrial raw materials on the natural environment has come under scrutiny. One of the classes of industrial raw materials that we found most interesting was preservatives. A commonly used preservative, formaldehyde, had recently been reclassified by the International Agency for Research on Cancer (IARC) from "probably carcinogenic to humans" to "carcinogenic to humans." The aim of the first part of this study was to synthesize alternative preservatives to formaldehyde. The isothiazolinone backbone was chosen and was achieved by first producing the *Z*-adduct from the base catalyzed reaction between propynoic acid and toluene-α-thiol to produce (*Z*)-3-benzylsulfanylpropenoic acid in a yield of 74%. (*Z*)-3-benzylsulfanylpropenoic acid was then activated with diphenylphosphinic chloride to furnish the phosphinic ester, which was not isolated but allowed to react directly with a range of amines to produce the corresponding amides. These amides were then allowed to react with 3-chloroperbenzoic acid in dichloromethane to furnish the corresponding sulfoxides. The sulfoxides were then treated with trichloroacetic anhydride to furnish the corresponding *N*-alkyl-isothiazol-3(2*H*)-ones. *N-(2-ethylphenylisothiazol-3(2H)-one* was successfully produced in a yield of 56%. The second part of the study involved the revival of gram positive (*B. pumilus*) and gram negative (*Citrobacter freundi*) bacterial strains as well as a sewage consortium that were previously isolated. This was successfully achieved using tryptone soy broth as the growth medium at a temperature of 30 °C over 24 hours. The third part of the study was to select a range of preservatives (diazolidinyl urea, imidazolidinyl urea, formaldehyde and the isothiazolinone derivatives) and evaluate the biodegradation of these raw materials under conditions that simulated that of a natural wastewater treatment plant over a period of 50 days. The difference in biodegradation between the sewage consortium and pure strains of gram positive (*B. pumilus*) and gram negative (*Citrobacter freundii*) bacteria was then assessed. Formaldehyde and the isothiazolinone derivative, *N-(2-*

ethylphenylisothiazol-3(2H)-one, could not be detected in the experimental medium and therefore no results were available. Diazolidinyl urea was best biodegraded by gram negative bacteria, *citrobacter freundii*, showing a percentage biodegradation of 51.4% whereas imidazolinyl urea was best biodegraded by the sewage consortium, showing a percentage biodegradation of 9.0%.

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CHAPTER 1

INTRODUCTION

1.1. Biodegradation and Green Chemistry Technologies

Biodegradation refers to the ability of a substance to decompose or breakdown under natural conditions into elements found in nature. A major contributor to world economic development is the chemical industry and it has become a key player in the introduction of future technologies. However, the negative impact that chemical usage has had on the environment has led to the development of green chemistry technologies.[1] Green Chemistry technologies aim to eliminate or at least reduce the risks of chemical usage to an acceptable level. The risk of chemical usage can be defined as the hazards of a particular chemical component associated with the exposure to these hazards.[1] Existing legislation with respect to risks have sought to deal with limiting exposure to these risks, whereas green chemistry has sought to minimize hazards in contrast to exposure. Therefore green chemistry aims to design or select chemicals with reduced toxicity and to choose reaction pathways that eliminate by-products and ensure the release of benign products. In recent years, the reduction of hazards has become an important criterion in industry for judging the performance of a product. Chemical products should be designed to preserve product efficacy while reducing toxicity. In addition, recent times and green chemistry has created much emphasis on biodegradation. [1, 2]

The biodegradation of raw materials entering wastewater treatment plants has become an important criterion for the classification of a product as 'environmentally friendly.' It was noticed at first in the 1960's that sewage treatment problems were arising. The amount of foam on rivers was increasing rapidly and it was discovered that propylene-based alkyl benzene sulfonates were not being completely degraded by bacteria naturally present in effluents. The branched chain formation of the alkyl benzene was thought to hinder bacterial attack on the chain. It was found however, that fatty acid sulfates degraded very easily and were produced of the straight chain variety. Proof that straight chain alkyl benzenes was biodegradable as opposed to branched chain alkyl benzenes, prompted legislation to be introduced prohibiting the discharge of non biologically degradable material into the sewer system.[3]

1.2. The fate and potential impact of industrial raw materials

Ecotoxicology is an interdisciplinary science concerned with investigating and quantifying the environmental fate and possible adverse effects of pollutants.[4] Holdgate (1979) defined pollution as "the introduction by man into the environment of substances or energy liable to cause hazards to human health, harm to living resources and ecological damage or interference with legitimate uses of the environment."[5] Considering the broadness of the statement made by Holdgate, industrial raw materials very accurately fit the description of being defined as a pollutant. It has estimated worldwide that over 63 000 chemicals are in common use and that between 200 and 1000 new synthetic chemicals are marketed each year.[6] For many of these substances, little, if anything, is known about their possible ecological effects.[5] To be useful, prior to the release of the compound into the market, both the toxicity of the compound and the proportion that may be

leaked or directly released into the environment, as well as the probable environment fate needs to be determined. The extent of releases and their possible ecological effects must be considered throughout the foreseeable life cycle of the product. This should include and assessment of the threats posed by degradation products and the ultimate disposal of the original product.[5] An overview of the fate of industrial raw materials is shown in Figure 1, outlining both the direct and indirect methods of release of pollutants into the environment.

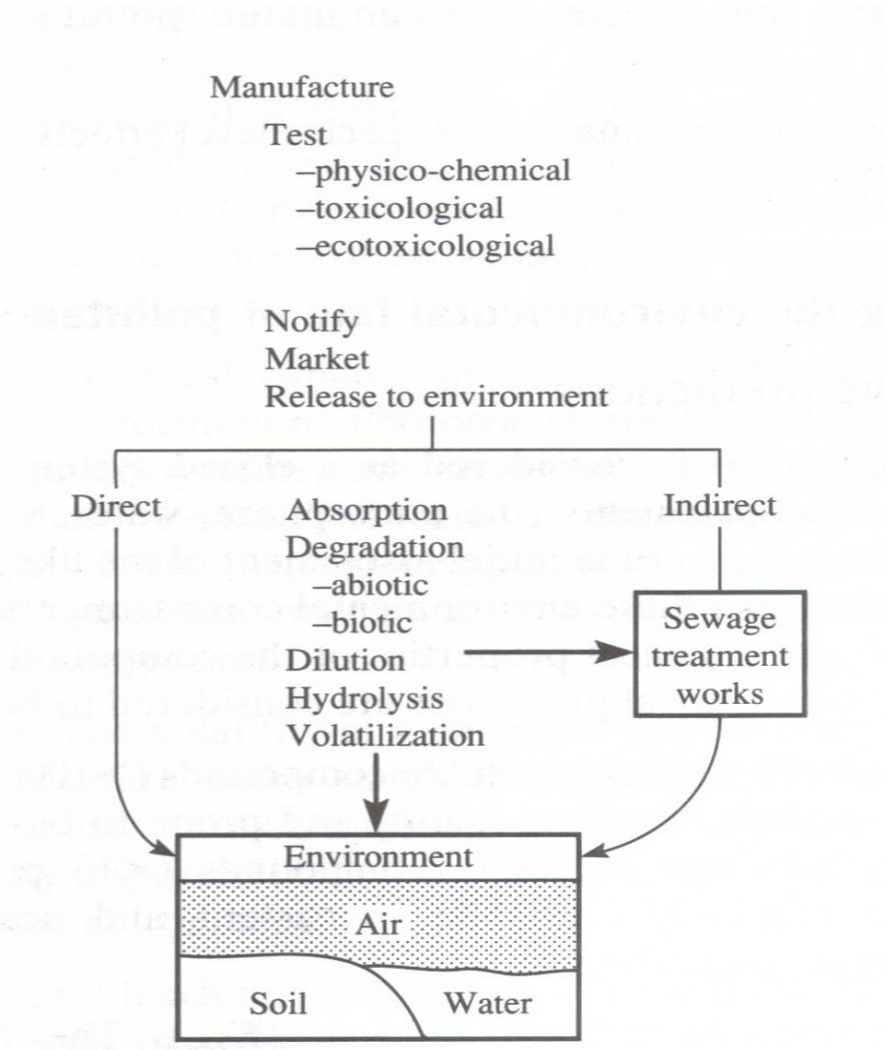


Figure 1: Pathway showing the release of chemicals into the environment both directly and indirectly[5]

1.3. Preservatives

1.3.1. The history of preservatives

In the 13th century, Roger Bacon, an English friar, claimed that "invisible creatures" caused disease. It was not until the late 17th century that a wealthy linen merchant, Anton van Leeuwenhoek, developed the microscope and revealed the existence of an entire universe of tiny living creatures. He was the first to see microorganisms. Because of his careful drawings of these microorganisms, most microbiologists consider him the Father of Microbiology.[7]

Microbiology was primarily a curiosity for 200 years after van Leeuwenhoek's discovery and did not explode into a scientific discipline until the late 1800s. This surge in interest was mainly due to the spontaneous generation controversy and Koch's postulates for infectious disease. In the 20 years from 1880 to 1900 — the Golden Era of microbiology — scientists discovered causative organisms of almost every important disease. They isolated, described, and controlled the major bacterial pathogens and even a few viruses. Thus began the taming of killers such as anthrax, diphtheria, tetanus, typhoid fever, yellow fever, rabies, syphilis, and tuberculosis.[7]

An explosion of research and studies lead to the Uniform methods and taxonomic descriptions becoming standards. The major contributors were the American Public Health Association (APHA) and the Society of American Bacteriologists (SAB). The American Public Health Association (APHA) published the first editions of Standard Methods for Water and Standard Methods for Dairy Products in the early 1900s. The first edition of Recommended Methods for Microbiological Examination of Foods was published in 1958. This was the beginning of product preservation and the introduction of preservatives into industry.[7]

Preservatives are by their very nature toxic and are designed to kill microorganisms. There are many different classes of preservatives and their classification is based on the types of agents that they contain. Most preservatives are toxic to the environment but in recent years, several companies have developed botanically based preservatives that are believed to be 99.9% effective against common germs, yet are non-toxic to humans and safe to use in the environment. In addition, it is also important to realize that different microorganisms vary considerably in their sensitivity and resistance to preservatives. [8]

Table 1: Classes of preservatives[8]

Class of Preservative	Examples of classes
Alkalies	Hydroxides
Biguanides	Chlorhexidine
Cationic surfactants	Quaternary Ammonium Compounds (QAC's)
Halogens and Halogen-containing compounds	Chlorine- based
Iodine-based	Iodophors
Oxidizing agents	Peroxides
Phenols and related compounds	Phenolics
Reducing Agents	Aldehydes

Effective preservatives exhibit the following properties:[9]

- provide a broad spectrum of proven activity.
- demonstrate a reduction in viable organisms for which it comes into contact with.
- work in a broad temperature range and in a variety of pH conditions.
- work in the presence of organic material such as urine, faeces or blood.

One of the most common industrial disinfectants that meets the above criteria and is cost effective as well is formaldehyde.[8]

1.3.2. Carcinogenicity of industrial preservatives

One of the recent industrial preservatives that have come under scrutiny is formaldehyde better known as formalin. Formaldehyde is an extensively used chemical and its ill effects have been of concern. Its nephrotoxic effects on laboratory animals and carcinogenic effects on humans have been well established.[10] Formaldehyde is a high production volume chemical with a wide array of uses.[11] Given its economic importance and widespread use, many people are exposed to formaldehyde environmentally and/or occupationally.[12] Presently, the International Agency for Research on Cancer classifies formaldehyde as carcinogenic to humans (Group 1), based on sufficient evidence in humans and in experimental animals.[12,13] Manyfold *in vitro* studies clearly indicated that formaldehyde can induce genotoxic effects in proliferating cultured mammalian cells.[12] Furthermore, some *in vivo* studies have found changes in epithelial cells and in peripheral blood lymphocytes related to formaldehyde exposure.[12] Methylparaben and propylparaben are also commonly used preservatives and have been proven to be non carcinogenic. Preservatives that are commonly referred to

as formaldehyde donors are diazolidinyl urea and imidazolidinyl urea and may possibly become the subject for further study.

1.3.3. Parabens

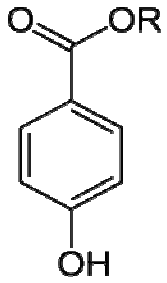


Figure 2: General structure of parabens[14]

Parabens are a group of chemicals widely used as preservatives in the cosmetic and pharmaceutical industries. Parabens are effective preservatives in many types of formulations. These compounds, and their salts, are used primarily for their bacteriocidal and fungicidal properties. They can be found in shampoos, commercial moisturizers, shaving gels, cleansing gels, personal lubricants, topical/ parenteral pharmaceuticals and toothpaste. They are also used as food additives.

1.3.3.1. Methylparaben

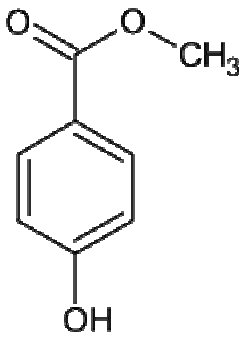


Figure 3: Chemical structure of methylparaben[15]

Methylparaben is used as a antimicrobial preservative in food, drugs, and cosmetics.[16] The compound is often found in carpules of local anaesthetic, acting as a bacteriostatic agent and preservative. Methylparaben is a methyl ester of *p*-hydroxybenzoic acid.[16] It is readily and completely absorbed through the skin and from the gastrointestinal tract. It is then hydrolyzed to *p*-hydroxybenzoic acid, conjugated and the conjugates are rapidly excreted in the urine.[16] Sensitization to methylparaben has occurred when medications containing parabens have been applied to damaged or broken skin.[16] Allergic reaction to ingested methylparaben have also been reported.[16]

1.3.3.2. Propylparaben

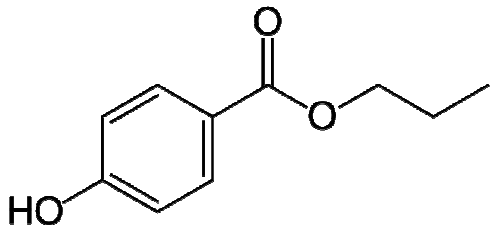


Figure 4: Chemical structure of propylparaben[16]

Propylparaben, the propyl ester of *p*-hydroxybenzoic acid, occurs as a natural substance found in many plants and some insects, although it is manufactured synthetically for use in cosmetics, pharmaceuticals and foods.[17] It is a preservative typically found in many water-based cosmetics, such as creams, lotions, shampoos and bath products.[17]

1.3.4. Formaldehyde

1.3.4.1. History of formaldehyde

Formaldehyde is the simplest aldehyde with the chemical formula CH_2O. Since its accidental production by Butlerov in 1859 and subsequent discovery by Hofmann in 1868, formaldehyde has become a major industrial product. Hofmann passed a mixture of methanol and air over a heated platinum spiral and then identified formaldehyde as the product. This method lead to the major way in which formaldehyde is manufactured today, the oxidation of methanol with air using a metal catalyst.[16]

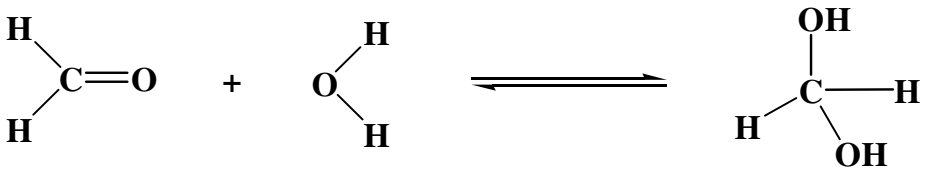
Bakelite, the first synthesized plastic, is the trademark name for the polymer of formaldehyde and phenol whereas Formica is the trademark name for the polymeric mixture of formaldehyde and urea. It is a resin used widely as glue, fire retardant and water repellent.

$$2\,CH_3OH\,(g) + O_2\,(g) \xrightarrow{\text{Catalyst } (450^0C - 650^0C)} 2\,CH_2O\,(g) + 2H_2O$$

Processes for production of formaldehyde from methanol were first developed and are still the major source of commercial formaldehyde. The reaction above demonstrates how this occurs. Catalysts used include copper, molybdenum alloy, platinum or silver.

Commercially, formaldehyde is manufactured in the form of an aqueous solution containing 37% by weight of dissolved formaldehyde. In this form, it is called formalin. Unless a solvent such as 1% methanol is used, formaldehyde will

polymerise at higher concentrations. Heating is also required to prevent precipitation of the polymer.[18]



Formalin

1.3.4.2. Reclassification of formaldehyde

Environmental concerns about the use of formaldehyde in industry have emerged relating to the effects of over exposure of formaldehyde.[19,20,21] Initial investigations on formaldehyde have resulted in its classification according to the International Agency for Research on Cancer (IARC) as "probably carcinogenic to humans." The IARC has re-evaluated formaldehyde and following this, formaldehyde was reclassified from Category 2A which states that it is "probably carcinogenic to humans" to Category 1 which states that it is "carcinogenic to humans." Following the IARC investigations, sufficient evidence was available to conclude that there is an epidemiological association of occupational work place exposure to formaldehyde with some increase in nasopharyngeal cancer, a rare cancer in developed countries.[22, 23, 24] However, there is insufficient evidence to associate formaldehyde exposure with any other forms of cancer.[13]

1.3.5. Formaldehyde donors

Formaldehyde donors have received much publicity due to the initial investigations relating to formaldehyde referring to its carcinogenic nature.[25, 26, 27] Two of the most commonly used formaldehyde donors in industry are diazolidinyl urea and imidazolidiniyl urea.[28, 29]

1.3.5.1. Diazolidinyl Urea

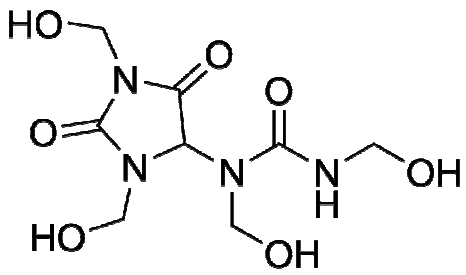
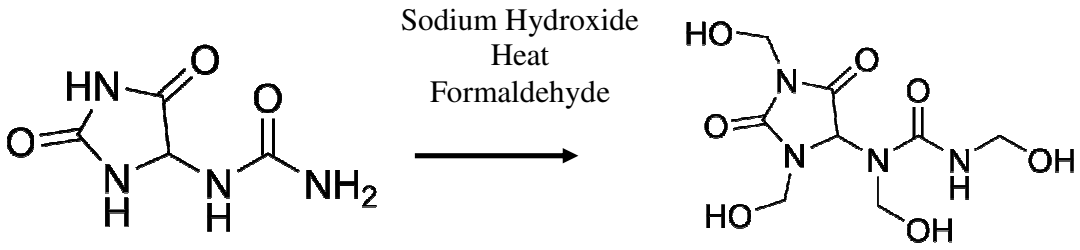


Figure 5: Chemical structure of diazolidinyl urea[30]

Diazolidinyl urea is used as an antimicrobial agent within the cosmetic industry.[31] Diazolidinyl urea is also a known formaldehyde releaser. It is used in a wide range of cosmetic products. It is produced by the reaction between formaldehyde and 2,5-dioxo-4-imidazolidinyl urea and heated in the presence of sodium hydroxide.



2,5-dioxo-4-imidazolidinyl urea **Diazolidinyl urea**

Figure 6: Conversion of 2,5-dioxo-4-imidazolidinyl urea to diazolidinyl urea

1.3.5.2. Imidazolidinyl Urea

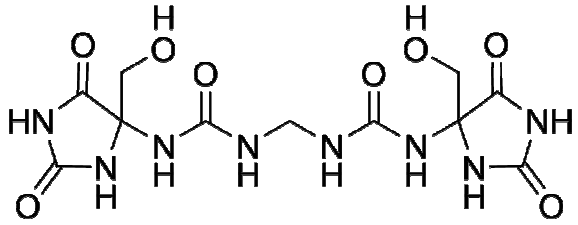
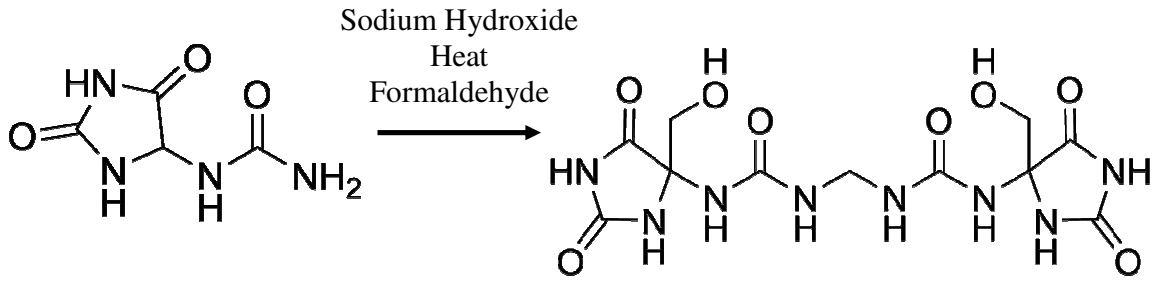


Figure 7: Chemical structure of imidazolidinyl urea[32]

Imidazolidinyl urea is used as a antimicrobial agent within the cosmetic industry. It is also known as a formaldehyde releaser. Imidazolidinyl urea is produced by reacting 2,5-dioxo-4-imidazolidinyl urea with formaldehyde in the presence of sodium hydroxide and heat. The reaction is then neutralized with hydrochloric acid.

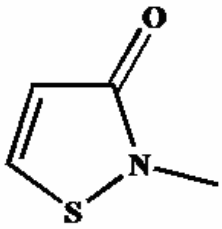


2,5-dioxo-4-imidazolidinyl urea **Imidazolidinyl urea**

Figure 8: Conversion of 2,5-dioxo-4-imidazolidinyl urea to imidazolidinyl urea[32]

1.3.6. Isothiazolinones

1.3.6.1. Methylisothiazolinone



Methylisothiazolinone or MIT, is a powerful biocide and preservative within the group of isothiazolinones, used in shampoos and body care products. Though long considered safe for use in cosmetics, two recent *in vitro* studies have shown that MIT is neurotoxic, causing damage to rat brain cells in tissue culture. Long-term health and safety studies have been conducted on animals, and thus far there is no published evidence of nerve damage or neurological effects associated with MIT

for consumers or workers.[33] None of the animal safety studies, however, are published in the primary scientific literature in peer-reviewed journals. Regulatory authorities in the USA, Japan and Europe and more than 25 other countries have all independently concluded the product is safe. Despite these claims, the studies published in scientific journals suggest that additional testing may be needed. Initially, a similar conclusion was reached by the European Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers in 2003. However, in 2004, after receiving additional studies, the European Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers concluded "The SCCNFP is of the opinion that the proposed use of methylisothiazolinone as a preservative at a maximum concentration of 0.01% (100 ppm) in the finished cosmetic product does not pose a risk to the health of the consumer." Whether methylisothiazolinone poses a risk via other forms of exposure or in occupational settings during the manufacture of products containing the biocide has yet to be determined.

1.4. Biodegradation of Preservatives

Since the beginning of the 20th century, it has been known that microorganisms have the ability to decompose aromatic compounds and many of them with intrinsic antimicrobial power. Some of the early work conducted on the biodegradation of preservatives highlighted that biocides found in soil seemed to disappear from the soil and could not be detected. It was then concluded that these compounds were being metabolized by soil microorganisms. This finding gave rise to extensive biochemical investigations into the mechanisms of decomposition and degradation of biocides. This finding also attributed to the fact that due to the decomposition of biocides by microorganisms, many preservative systems fail.[34]

1.5. Waste Water Treatment

A waste water treatment is the process of removing physical, chemical and biological contaminants from waste water effluents and domestic sewage. A series of physical, chemical and biological processes are included. The purpose of waste water treatment is to produce an environmentally safe treated effluent and solid waste known as sludge suitable for disposal or reuse in most cases as farm fertilizer.

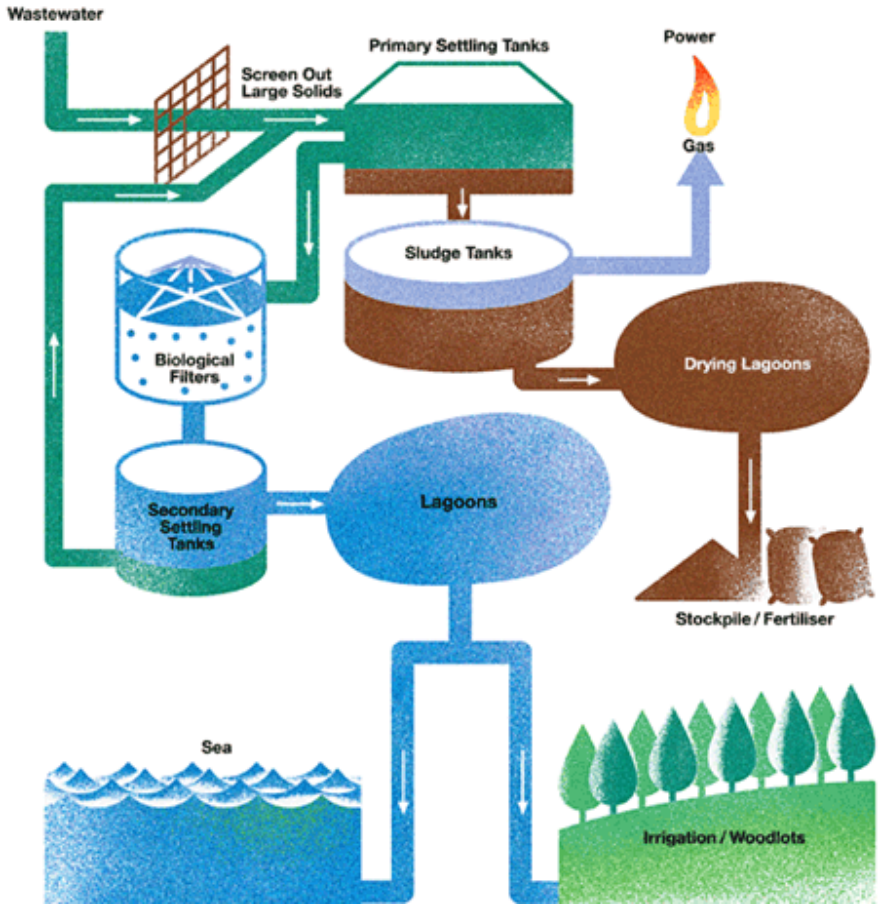


Figure 9: Wastewater treatment plant[35]

Waste water treatment usually involves three stages:

- **Primary treatment**- involves allowing heavy solids to settle to the bottom whilst allowing the lighter solids to float to the top along with the oil and grease. The settled materials are removed and the remaining liquid subjected to secondary treatment.

- **Secondary treatment**- involves the process of removing dissolved and biological matter.[36,37] This is possible by using microorganisms in a controlled habitat. A separation process of the microorganisms from the treated water is necessary before being subjected to tertiary treatment. Figure 10 below presents the secondary treatment plant at Bushkoppies waste water treatment plant in Johannesburg.

Figure 10: Overview of wastewater treatment plant containing waste activated sludge.

- **Tertiary treatment**- the tertiary treatment stage involves disinfecting any water that may be released into the ecosystem.

For the purposes of this study we were particularly interested in the secondary treatment stage containing the activated sludge. At this stage, processes involving the use of dissolved oxygen to promote the growth of microorganisms that removes organic material. The activated sludge is therefore rich in a wide consortium of bacterial strains that would be useful in helping us simulate the conditions of the waste water treatment plant.

1.5.1. Bacterial composition

Activated sewage sludge is treated using aerobic biological processes. The microorganisms used require both oxygen and nutrients to survive. The sludge therefore provides a rich nutrient medium for the microorganisms and allows them to sustain growth therefore referring to the secondary treatment phase as a suspended growth system.

1.6. Bacteria

Bacteria as biodegraders have the ability to utilize all naturally occurring compounds as sources of energy and carbon.[38, 39] The capability of microbes has evolved over 3 billion years of the planets history. Microbial degradation has been used in technologies for the degradation of natural household waste by the use of septic tanks and sewage works.[40,41,42] Research has shown that bacteria are extremely genetically adaptable and due to their rapid growth rates have a range of

mechanisms that allow them to adapt to new environments.[43] The bacterial cell wall plays an important role in acting as a barrier to biocides and protects the cell against the external environment.[44] It also helps to maintain the cell shape and maintain the cell integrity. Internal to the cell wall is the cell membrane, cytoplasm, mesosome, ribosome, nucleoid and inclusion body. Figure 11 below represents a typical bacterial cell.

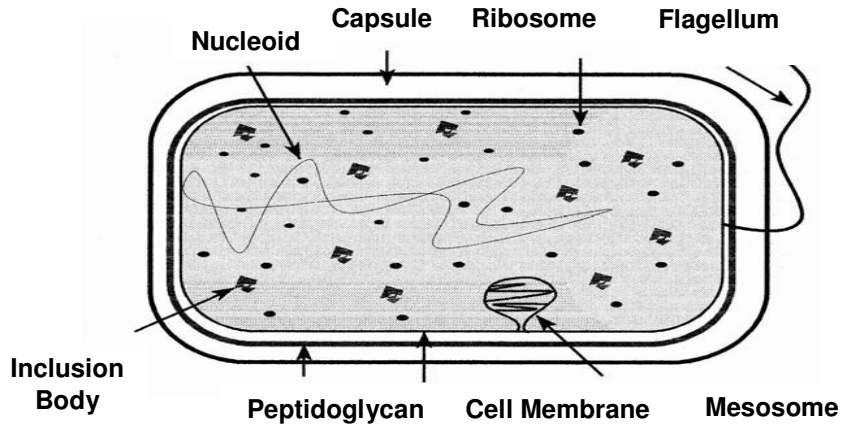


Figure 11: Bacterial cell structure

1.6.1. Gram positive bacteria

Gram positive bacteria are represented by the dark purple crystal violet stain retained by the thick layer of peptidoglycan which forms the outer later of the cell. The following characteristics are present in gram positive bacteria:[43]

- a cytoplasmic lipid membrane
- a thick peptidoglycan layer containing teichoic acid and lipoteichoic acid

Figure 12 below represents the cell wall structure of gram positive bacteria.

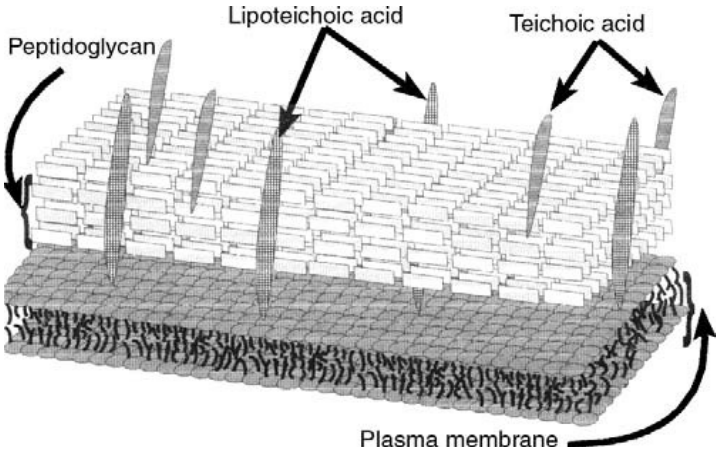
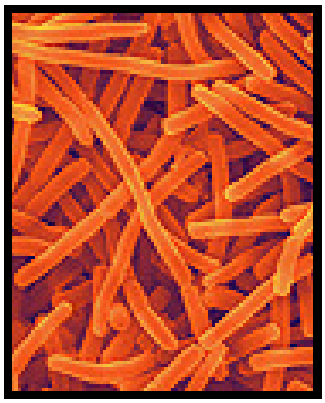


Figure 12: Cell wall structure of Gram positive bacteria



Bacillus pumilus

Figure 13: Image of Gram positive bacteria (*B. pumilus*)

It has been repeatedly found that species of extremely resilient, spore forming bacteria belong to the genus Bacillus.[45, 46] Members of the genus Bacillus and related genera are ubiquitous in nature.[47] *B. pumilus*, is a ubiquitous gram-positive, aerobic, rod-shaped endospore-forming bacteria that can be isolated from a wide

variety of soils, plants and environmental surfaces.[48] *B. pumilus* was found to the most resistance when compared to any other bacillus species.

1.6.2. Gram negative bacteria

Gram negative bacteria do not retain the crystal violet dye as per the gram staining protocol.[49] A counter stain is usually used after the crystal violet stain colouring all gram negative bacteria red or pink. The following characteristics are present in gram negative bacteria:

- a cytoplasmic membrane
- a thin peptidoglycan layer
- a periplasmic space
- no teichoic acids or lipoteichoic acids are present
- Braun's lipoproteins

Figure 14 below represents the cell wall structure of gram negative bacteria.

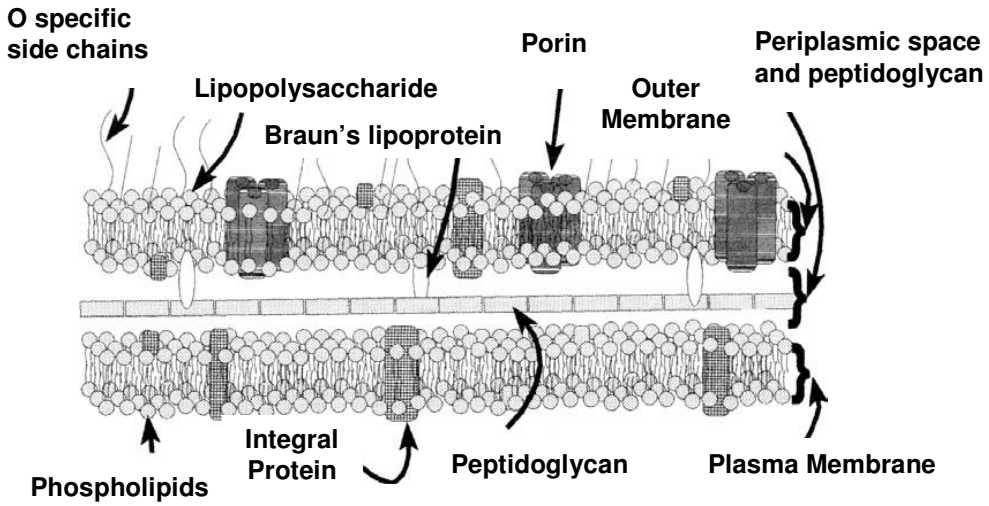


Figure 14: Cell wall structure of gram negative bacteria

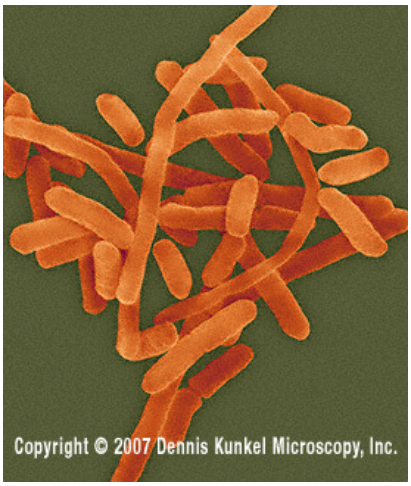


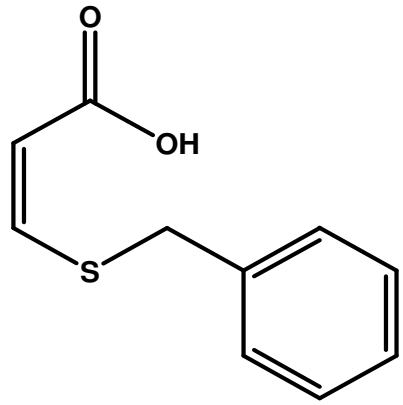
Figure 15: Image of gram negative bacteria (*Citrobacter freundii*)

Citrobacter freundii is an example of gram negative bacteria. These bacteria can be found almost everywhere in soil, water and waste water. They can also be found in the human intestine, but are rarely the cause of any illness.[50, 51]

1.7. Project Aims

1.7.1. Synthesis of Isothiazolinone Derivatives

The aim of this project was to synthesize alternatives to formaldehyde as a preservative that is used widely in industry, in a range of products available to consumers. Using an extensive literature review we attempted the synthesis of the well known isothiazolinone family, and derivatives thereof, via a trichloroacetic acid-mediated ring closure of *N*-substituted (*Z*)-3-(benzylsulfinyl)propenamides. The first step was to produce *3-(Z)-(Benzylsulfanyl)propenoic acid* **[1]** which then underwent a series of reactions to yield the isothiazolinone structure. Commonly used in industry are the methylisothiazolinones **[2]** and methylchloroisothiazolinones **[3]**.



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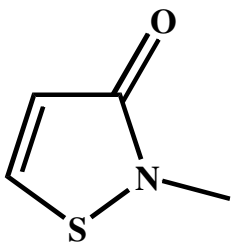

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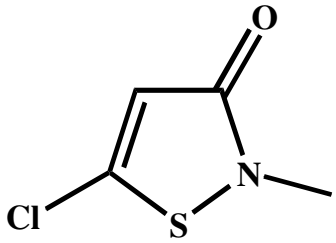


3

1.7.1.1. Envisaged route by Haefliger and Pertrizilka

Using the methodology set out by Haefliger and Petrizilka, *3-(Z)-(Benzylsulfanyl) propynoic acid* **[1]** was produced via a base catalysed reaction of toluene-α-thiol and propynoic acid. The addition of thiols to alkynes furnished *Z*-adducts under base catalyzed conditions whereas the non-catalyzed system is known to furnish *E*-sulfides. We were particularly interested in the *Z*-adducts which were then used to generate a range of amides required for synthesis of the isothiazolinone structure.

1.7.1.2. Envisaged route by Harwood and co-workers

With the *3-(Z)-(Benzylsulfanyl) propenoic acid* **[1]** in hand, we attempted the synthesis of a range of amides using diphenylphosphinic chloride to furnish the phosphinic ester which was allowed to react with a range of amines and furnish the respective amides. The respective amides were then converted into the corresponding sulfoxides using 3-chloroperbenzoic acid. These sulfoxides then underwent a trichloroacetic acid-mediated ring closure to yield the corresponding *N*-alkyl-isothiazol-3(2*H*)-ones.

1.7.2. Bacterial Culture Growth

We attempted to revive bacterial cultures isolated form previous biodegradation experiments using tryptone soy broth as a nutrient rich medium. The cultures were then plated to ensure growth and isolated for use in a biodegradation experiment. Two strains of gram positive and gram negative bacteria were revived as well as a sewage bacteria consortium. This combination of bacteria was chosen to determine

the extent of breakdown individual strains may cause and also bearing in mind the differences between these strains, the speed at which they breakdown the selected components.

1.7.3. Biodegradation of selected industrial preservatives

We selected some of the most commonly used industrial preservatives that were both cost effective and worked efficiently to minimize bacterial growth in products available to consumers. Once the selection was made, we set up a medium into which they were added and inoculated with a specific strain of bacteria or the sewage consortium. We then assessed the extent of biodegradation of these preservatives and the potential damage it may cause to the environment if not biodegraded and released into natural waste water treatment plants.

CHAPTER 2

EXPERIMENTAL PROCEDURES

2.1. General Experimental Procedures

2.1.1. Chromatographic Separations

The Rf values quoted are for analytical thin layer chromatography (TLC) on aluminium-backed Macherey-Nagel Alugram Sil G/UV_{254} plates pre-coated with 0.25 mm silica gel 60, detected by UV absorption.

Preparative column chromatography was carried out on both wet- and dry-packed columns using Macherey-Nagel Kieselgel 60 silica gel 60 (particle size 0.063-0.200 mm) as the adsorbent. Mixtures of ethyl acetate and hexane were used as the mobile phase.

2.1.2. Spectroscopic and Physical data

All melting points were obtained on a Reichert hot-stage microscope, and are uncorrected.

^{1}H NMR (Nuclear Magnetic Resonance) spectra were recorded on a Bruker AVANCE 300 (300.13 MHz) or a Bruker 400 (400.14 MHz) spectrophotometer. Spectra were recorded in deuterated chloroform ($CDCl_3$) and chemical shifts are reported in parts per million downfield from tetramethylsilane, the internal

standard; coupling constants are given in Hertz. Splitting patterns are designated as "s", "d", "t", "q" and "m"; these symbols indicate "singlet", "doublet", "triplet", "quartet" and "multiplet" respectively. NMR data are reported as follows: chemical shift (integration of signal, description of signal, assignment).

^{13}C NMR (1H decoupled) spectra were recorded on a Bruker AVANCE 300 (75.47 MHz) or Bruker DRX 400 (100.63 MHz) spectrophotometer. Spectra were recorded in deuterated chloroform ($CDCl_3$) and chemical shifts are reported in parts per million relative to the central signal of deuterated chloroform, taken as δ 77.00.

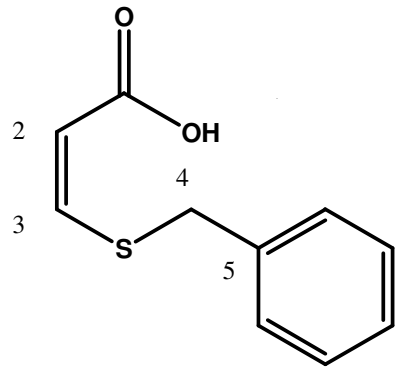
IR (infrared) spectra were recorded on a Bruker IFS-25 Fourier Transform spectrophotometer or on a Bruker Vector-22 Fourier Transform spectrophotometer. Liquid samples were recorded as thin film between sodium chloride plates, while solid samples were recorded as solutions in chloroform in sodium chloride cells. The signals were reported on the wavenumber (ν/cm^{-1}). Signals were designated as "s", "m", "w" and "br"; these symbols indicate "strong", "medium", "weak" and "broad" respectively. IR spectra data were reported as follows: wavenumber (intensity, assignment).

2.1.3. Other General Procedures

The term "*in vacuo*" refers to the removal of solvent by rotary evaporation, followed by removal of the residual solvent at oil pump pressure (ca. 0.1-1 Torr) at ambient temperature until constant mass was achieved.

2.2. Part A- Synthesis of Isothiazolinone Derivatives

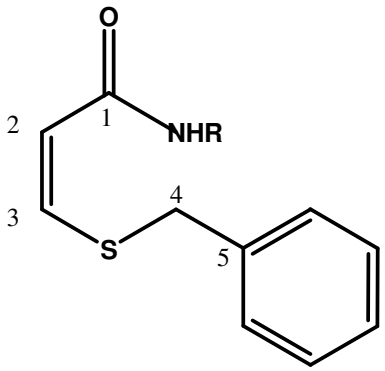
2.2.1. Synthesis of *3-(Z)-(Benzylsulfanyl) propenoic acid*



Propynoic acid (10.6 g, 150 mmol, 1 eq) was added to a solution of sodium carbonate (17.5 g, 165 mmol, 1.1 eq) and distilled water (200 cm^3). Methanol (150 cm^3) was then added to the mixture and allowed to stir for 5 minutes. Toluene-α-thiol (17.6 cm^3, 150 mmol, 1 eq) was then added to the resulting mixture and refluxed for 4 hours. The solution was then stirred at room temperature for 24 hours allowing sufficient time for completion of the reaction. The mixture was then acidified with 2 mol dm^3 hydrochloric acid until a pH of 3 was reached. This was then extracted with ethyl acetate (3 x 100 cm^3). The organic layer was then washed with distilled water (3 x 100 cm^3) and brine (3 x 100 cm^3, dried with $MgSO_4$ and concentrated under reduced pressure. The product was purified by recrystallization (acetone-hexane) to yield colorless crystals (21.6 g, 74%). $\mathbf{R}_f$ 0.75 (ethyl acetate: hexane; 6:4); m.p. 138-139 ^{0}C (lit[15], 138-139 ^{0}C); **IR** ($CHCl_3$): ν_{max} (cm^{-1}) = 3306 (s, br, OH), 1603 (s, C=O), 1533 (s, C=C), 1239 (s, C-S); **^{1}H NMR** (300 MHz, $CDCl_3$): δ_H = 9.50–11.50 (1H, br s, OH), 7.28-7.27 (5H, m, Ph); 7.19 (1H, d, *J*=10.2, 2-H), 5.84-5.81 (1H, d, *J*=10.23, 3-H), 3.96 (2H, s, 4-H), **^{13}C**

NMR (75 MHz, $CDCl_3$) δ_C = 172.0 (C=O), 152.0 (CH), 137.2 (C), 129.4 (CH), 129.2 (CH), 128.0 (CH), 113.2 (CH), 40.0 (5CH)

2.2.2. General procedure for the Synthesis of Amides



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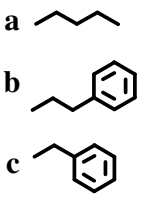


N-Methylmorpholine (2.2 eq) was added to a solution of *3-(Z)-(Benzylsulfanyl) propenoic acid* (1 eq) and THF (50 cm^3). This was then followed by the addition of diphenylphosphinic chloride (1.1 eq) to the mixture. The reaction was allowed to stir for 1 hour at $0^{\circ}C$ under a $CaCl_2$ drying tube. The required amine (1 eq) was then dissolved in THF (20 cm^3) and added drop-wise to the mixture. After addition of the amine to the solution, the reaction was allowed to warm to room temperature and monitored by TLC analysis for consumption of the amine. In some cases it was necessary to allow the reaction to take place over 24 hours. On completion of the reaction, the mixture was diluted with an equal amount of

diethyl ether and washed with a solution of 2 mol dm^3 sodium hydroxide. The organic layer was then washed with 1 mol dm^3 hydrochloric acid and brine, dried with $MgSO_4$ and concentrated under reduced pressure. The product was purified by recrystallization (ethyl acetate-hexane).

2.2.2.1. Synthesis of *(Z)-3-(Benzylsulfanyl)-N-butylpropenamide* 2a

Isolated as white crystals (194 mg, 76%); Rf 0.75 (ethyl acetate: hexane; 6:4); m.p. 85-86 ^{0}C; **IR** ($CHCl_3$) :ν_{max} (cm^{-1}) = 1640 (s, C=O), 1543 (s, C-NHR), 1256 (s, C-S); **^{1}H NMR** (300 MHz, $CDCl_3$): δ_H = 7.35-7.22 (5H, m, Ph), 6.79 (1H, d, *J*=12.0, 2-H), 5.72 (1H, d, *J*=12.0, 3-H), 5.47 (1H, br s, NH), 3.90 (2H, s, 4-H), 3.31-3.35 (2H, q, *J*=6.0, 4'-H), 1.53-1.44 (2H, m, 2'-H), 1.38-1.27 (2H, m, 3'-H), 0.925 (3H, t, *J*=6.0, 1'-H) ; **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C = 164.8 (C=O), 142.1 (CH), 136.3 (C), 127.7 (CH), 127.3 (CH_3), 125.9 (CH_2), 114.5 (CH), 76.1 (CH), 75.3 (CH), 38.3 (CH), 37.8 (CH), 30.4 (CH_2), 18.7 (CH_2), 12.4 (CH_2).

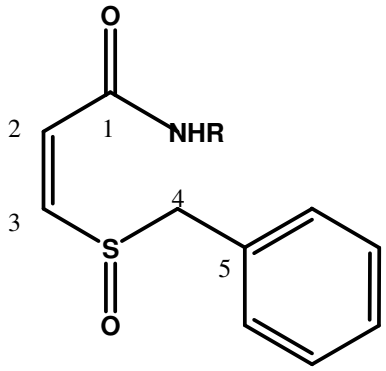
2.2.2.2. Synthesis of (*Z)-3-(Benzylsulfanyl)-N-benzylpropenamide* 2b

Isolated as white crystals (257 mg, 85%); Rf 0.85 (ethyl acetate: hexane; 6:4); m.p. 108-109 ^{0}C; **IR** ($CHCl_3$): ν_{max} (cm^{-1}) = 1639 (s, C=O), 1566 (s, C-NHR), 1257 (s, C-S) **^{1}H NMR** (300 MHz, $CDCl_3$): δ_H = 7.32-7.22 (5H, m, Ph), 7.22-7.15 (5H, m, Ph), 6.79 (1H, d, *J*=9.9, 2-H), 5.66 (1H, d, *J*=9.9, 3-H), 5.52 (1H, br s, NH), 3.89 (2H, s, 4-H), 3.57 (2H, q, *J*=6.8, 1'-H), 2.83 (2H, t, *J*=6.8, 2'-H); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C = 166.6 (C=O), 144.3 (CH), 139.4 (C), 138.0 (C), 129.4 (CH), 129.2 (CH), 129.1 (CH), 120.0 (CH), 127.7 (CH), 126.8 (CH_2), 116.1 (CH), 40.9 (CH), 40.0 (CH_2), 36.1 (CH_2).

2.2.2.3. ***(Z)-3-(Benzylsulfanyl)-N-(2-ethylphenyl)propenamide*** **2c**

Isolated as white crystals (256 mg, 88%); Rf 0.85 (ethyl acetate: hexane; 6:4); m.p. 93-94 ^{0}C ; **IR** ($CHCl_3$): ν_{max} (cm^{-1}) = 1637 (s, C=O), 1577 (s, C-NHR), 1260 (s, C-S); **^{1}H NMR** (300 MHz, $CDCl_3$): δ_H = 7.33-7.27 (5H, m, Ph), 7.27–7.25 (5H, m, Ph), 6.84 (1H, d, *J*=9.0, 2-H), 5.76 (1H, br s, NH), 5.74 (1H, d, *J*=9.0, 3-H), 4.46 (2H, d, *J*=3.0, 1'-H), 3.98 (2H, s, 4-H) **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C = 165.8 (C=O), 144.3 (CH), 138.2 (C), 137.4 (C), 128.9 (CH), 128.6 (CH), 128.5 (CH), 127.8 (CH), 127.3 (CH), 127.1 (CH), 115.3 (CH), 43.2 (CH_2), 39.4 (CH_2).

2.2.3. General procedure for the synthesis of sulfoxides



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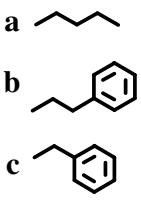


The required sulfide was dissolved in dichloromethane (5 cm^3 $mmol^{-1}$) to which, a solution of 3-chloroperbenzoic acid (1 eq) in dichloromethane was added drop wise at 0° C. The reaction mixture was stirred for 1 hour at 0^0C and monitored by TLC analysis. On completion of the reaction, the mixture was washed with a solution of saturated sodium hydrogen carbonate and brine, dried with MgSO4, and concentrated under reduced pressure. The product was purified by recrystallization (ethyl acetate-hexane).

2.2.3.1. ***(Z)-3-(Benzylsulfinyl)-N-butylpropenamide*** **3a**

Isolated as white crystals (137 mg, 71%); Rf 0.79 (ethyl acetate: hexane; 6:4); m.p.90-91 ^{0}C; **IR** ($CHCl_3$): ν_{max} (cm^{-1}) = 1676 (s, C=O), 1607 (s, S=O), 1503 (s, C-NHR), 1212 (s, C-S), **^{1}H NMR** (300 MHz, $CDCl_3$): δ_H = 7.37-7.25 (5H, m, Ph), 6.63 (1H, br s, NH), 6.56 (1H, d, *J*=12.0, 2-H), 6.26 (1H, d, *J*=12.0, 1-H), 4.11 (2H, q, *J*=12.0, 4-H) 3.22 (3H, q, *J*=12.0, 4'-H), 1.45 (2H, m, 2'-H). 1.27 (2H, m, 3'-H), 0.91 (2H, t, *J*=7.2, 1'-H) **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C = 163.5 (C=O), 162.6 (C), 152.4 (CH), 133.1 (CH), 131.1 (CH), 130.7 (CH_3), 130.6 (CH), 129.1 (CH_2), 128.5 (CH), 128.2 (CH), 127.2 (CH), 39.6 (CH_2), 20.0 (CH_2), 13.6 (CH_2).

2.2.3.2. ***(Z)-3-(Benzylsulfinyl)-N-benzylpropenamide*** **3b**

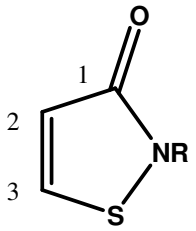
Isolated as white crystals (212 mg, 79%) ; Rf 0.12 (ethyl acetate: hexane; 6:4); m.p. 116-117 ^{0}C ; **IR** ($CHCl_3$): ν_{max} (cm^{-1}) = 1657 (s, C=O), 1605 (s, S=O), 1557 (s, C-NHR), 1283 (s, C-S); **^{1}H NMR** (300 MHz, $CDCl_3$): δ_H = 7.35-7.30 (5H, m, Ph), 7.30-7.19 (5H, m, Ph), 6.37 (1H, d, *J*=10.0, 2-H), 6.34 (1H, s, NH), 6.14 (1H, d, *J*=10.0, 3-H), 4.28 (2H, q, *J*=10.0, 4-H), 3.61 (2H, q, *J*=6.0, 1'-H), 2.88 (2H, t, *J*=6.0, 2'-H); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C = 163.4 (C=O), 138.7 (C), 138.3 (C),

130.7 (CH), 130.6 (CH), 129.6 (CH), 128.8 (CH), 128.7 (CH), 127.0 (CH), 126.7 (CH), 126.2 (CH_2), 40.9 (CH_2), 35.4 (CH_2).

2.2.3.3. *(Z)-3-(Benzylsulfinyl)-N-(2-ethylphenyl)propenamide* 3c

Isolated as white crystals (198 mg, 73%) Rf 0.79 (ethyl acetate: hexane; 6:4); m.p. 103-104 ^{0}C ; **IR** ($CHCl_3$): ν_{max} (cm^{-1}) =1685 (s, C=O), 1596 (s, S=O), 1555 (s, C-NHR), 1252 (s, C-S); **^{1}H NMR** (300 MHz, $CDCl_3$): δ_H = 7.39-7.25 (5H, m, Ph), 7.29-7.25 (5H, m, Ph), 6.38 (1H, d, *J*=10.0, 2-H), 6.35 (1H, br s, NH), 6.22 (1H, d, *J*=10.0, 3-H), 4.45 (2H, d, *J*=10.0, 1'-H), 4.18 (2H, q, *J*=6.0, 4-H) **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C = 162.6 (C=O), 158.3 (C), 138.3 (C), 130.8 (CH), 130.6 (CH), 129,6 (CH), 128.8 (CH), 128.6 (CH), 128.3 (CH), 127.0 (CH), 127.0 (CH), 126.8 (CH), 60.3 (CH), 40.9 (CH_2), 35. 4 (CH_2).

2.2.4. General procedure for the Synthesis of Isothiazolinones



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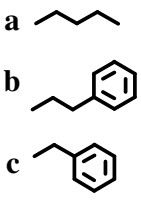


The required (*Z*)-3-(Benzylsulfinyl)-*N*-alkylpropenamide was allowed to dissolve in dichloromethane. The temperature was then reduced to $0^{\circ}C$ and the mixture placed under nitrogen. Trichloroacetic anhydride was then carefully added to the mixture that was allowed to stir for 2 hours at $0^{\circ}C$. The reaction mixture was then warmed up to room temperature and stirred for 24 hours. The reaction was monitored by TLC analysis. On completion of the reaction it was quenched with a solution of 2 mol dm^3 sodium hydroxide and extracted with dichloromethane. This was then dried with $MgSO_4$ and concentrated under reduced pressure. The product was then purified by column chromatography on silica gel by eluting first with dichloromethane and then a 30% ethyl acetate: hexane mixture.

2.2.4.1. *N-(2-Ethylphenyl)isothiazol-3(2H)-one* 4c

Isolated as a yellow oil (71 mg, 56%) Rf 0.19 (ethyl acetate: hexane; 6:4); **IR** ($CHCl_3$): ν_{max} (cm^{-1}) = 1643 (s, C=O), 1557 (s, C-NR), 1309 (s, C-S); **^{1}H NMR** (300 MHz, $CDCl_3$): δ_H = 8.08 (1H, d, *J*=3.0, 2-H), 7.39-7.25 (5H, m, Ph), 6.44 (1H, d, *J*=3.0, 3-H), 5.36 (1H, s, 1'-H), 4.96 (1H, s, 1'-H) **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C = 169.4 (C=O), 162.8 (C), 164.0 (CH), 137.3 (CH), 133.2 (CH), 131.1 (CH), 129.1 (CH), 140.3 (CH), 114.2 (CH), 47.9 (CH_2).

2.3. Part B- Bacterial Culture Growth

2.3.1. Preparation of Tryptone Soy Broth

30 g of soybean-casein digest medium USP (tryptone soy broth) was dissolved in 1 dm^3 distilled water. The mixture was stirred for 30 minutes until homogenous. The solution was then autoclaved at 121°C for 30 minutes to ensure that no microbial contamination was present so as to allow for controlled conditions during the experimental procedure.

2.3.2. Preparation of Tryptone Soy Agar Plates

38 g of soybean-casein digest agar medium USP (tryptone soy agar) was dissolved in 1 dm^3 distilled water. The mixture was then stirred for 30 minutes until homogenous. The solution was then autoclaved at 121°C for 30 minutes to ensure that no microbial contamination was present so as to allow for controlled conditions during the experimental procedure. The tryptone soy agar was then poured into sterile plastic petri dishes using aseptic techniques. The tryptone soy agar was then left for 24 hours in the plastic petri dishes to solidify.

2.3.3. Revival of bacterial cultures previously isolated

50 cm^3 tryptone soy broth was added to six 250 cm^3 conical flasks. The flasks were then sealed with foil and autoclave tape. The six flasks were then autoclaved for 30 minutes at 121°C and then allowed to cool. The frozen bacterial cultures (*B. pumilus, Citrobacter freundii, B. subtilis*, sewage bacteria consortium 1, sewage bacteria consortium 2) were then thawed out and inoculated into the flasks

respectively using aseptic techniques. The flaks were then sealed with foil and incubated at 30°C on a shaker for 24 hours.

2.3.4. Procedure for the Determination of Bacterial Growth

Using aseptic techniques, the bacterial cultures were then plated using a sterile loop onto agar plates previously prepared and incubated for 24 hours. Bacterial growth was then identified as compared to a control containing no bacterial culture.

2.4. Part C- Biodegradation of Selected Industrial Preservatives

2.4.1. Preparation of Minimal Medium

Trace salts solution: 23 mg $MnCl_2.2H_2O$, 30 mg $MnCl_4.H_2O$, 31 mg H_3BO_3, 36 mg $CoCl_2.6H_2O$, 10 mg $CuCl_2.2H_2O$, 20 mg $NiSO_4$, 30 mg $Na_2MoO_4.2H_2O$, 50 mg $ZnCl_2$ was dissolved in 1 dm^3 distilled water.

1M $MgSO4.7H_2O$: 12.32 g $MgSO_4.7H_2O$ was dissolved in 50 cm^3 distilled water.

2.5 cm^3 traces salts was mixed in 1 cm^3 1M $MgSO_4.7H_2O$ followed by 8.8 g $Na_2HPO_4.12H_2O$, 3 g KH_2PO_4, 1 g NH_4Cl and 0.5 g NaCl. The solution was then made up to 1 dm^3.[52]

2.4.2. Procedure for addition of selected industrial preservatives to minimal medium

Four experimental procedures were set up as represented by figure 16 below. Experiment 1 represented the biodegradation of diazolidinyl urea, experiment 2 represented the biodegradation of imidazolidinyl urea, experiment 3 represented the biodegradation of formaldehyde and experiment 4 represented the biodegradation of the synthesized organics compound. Sixteen Erlenmeyer flasks were prepared by adding 400 cm^3 minimal medium. The flasks were then autoclaved and allowed to cool. Further experimentation of the flasks was conducted out under aseptic conditions. 250 ppm of diazolidinyl urea, imidazolidinyl urea, formaldehyde and the synthesized organic compound was added to the cooled flasks containing minimal medium. The solution was then placed onto a shaker until completely homogenous. The chosen flasks were then inoculated with the sewage consortium, *B. pumilus* and *citrobacter freundii* respectively. The control contained no microbial contamination. See representation of experimental procedure below. The sixteen flasks were then placed onto a shaker at 190 rpm for 50 days.[53, 54, 55]

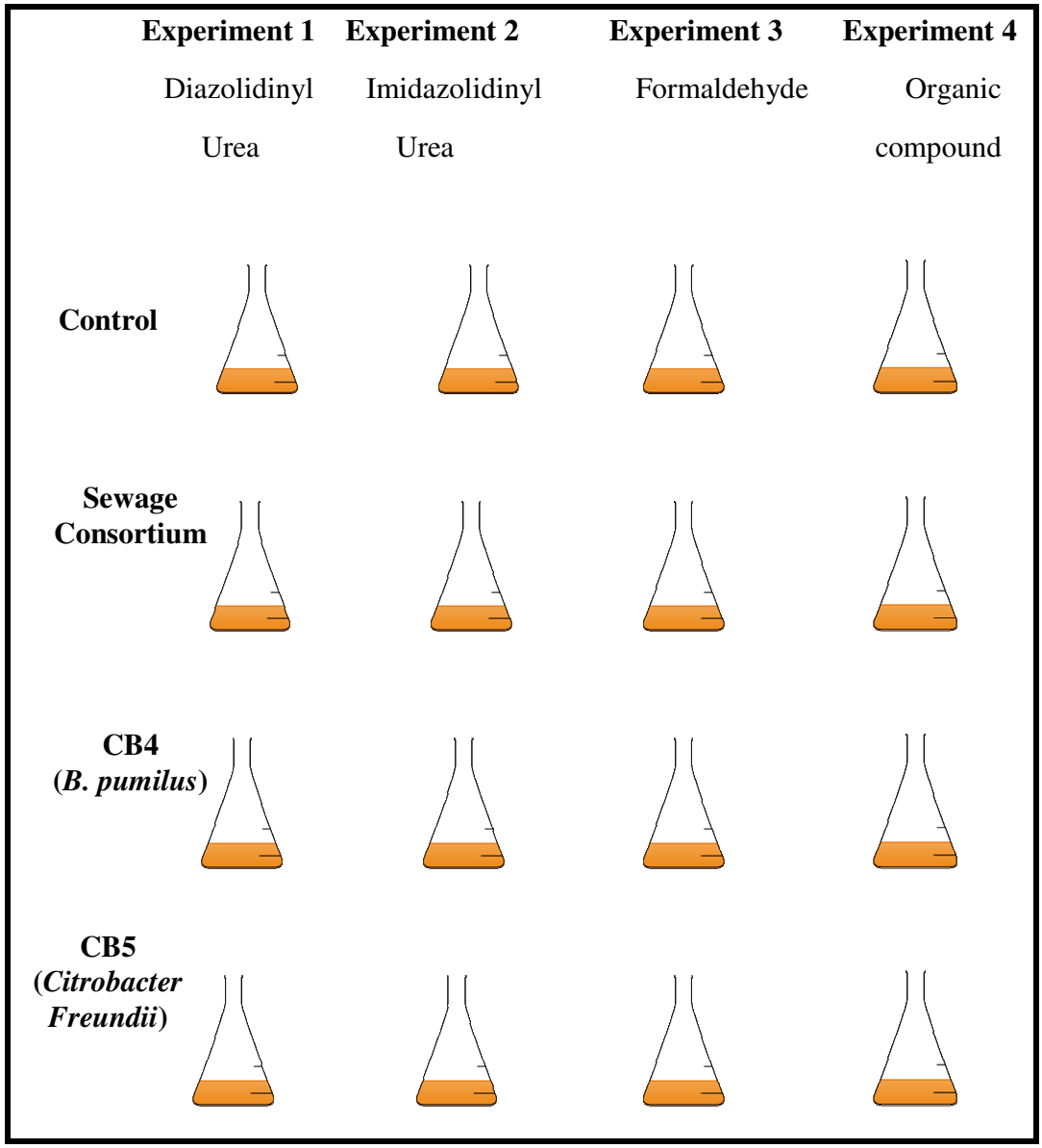


Figure 16: Representation of experimental procedure. [56]

2.4.3. Procedure for Toxicity Test

Using aseptic techniques, 3 cm^3 samples were then isolated from each flask at regular intervals. The optical density of each sample was then measured at 600 nm. The process involved calibrating the spectrophotometer with the control sample which contained no bacteria and therefore an absorbance reading of zero was set. The absorbance of the experimental sample was then determined.

2.4.4. Procedure for Extraction of Biodegraded Components

3 cm^3 of experimental sample was removed from each flask at regular intervals and filtered to remove any bacteria.

2.4.5. Procedure for HPLC Analysis

A C18 column was used for the analysis of each of the experimental samples. The mobile phase used was 60% deionised water: 40% acetonitrile. Standards of 50 ppm, 100 ppm, 150 ppm, 200 ppm and 250 ppm on each of the compounds were run and once each of the experimental samples were isolated and filtered, 10 µl was injected into the HPLC. [57]

CHAPTER 3

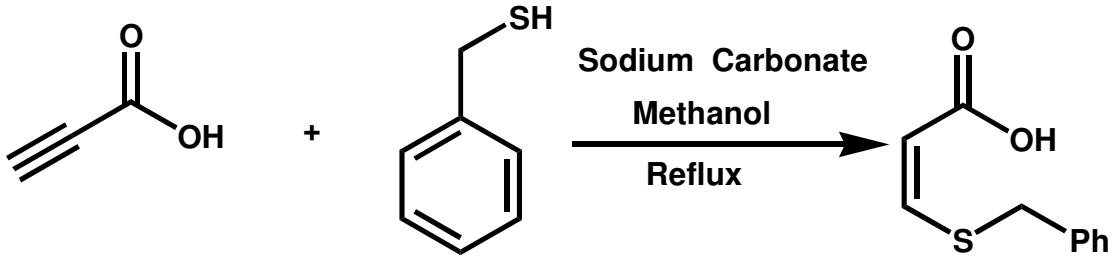
RESULTS AND DISCUSSIONS

3.1. Part A- Synthesis of Isothiazolinones Derivatives

Following an extensive literature review on the synthesis on isothiazolinones and their derivatives, we settled on the route as determined by Harwood and co-workers. Initial steps to attaining the final product was to first synthesize 3-(*Z*)-(benzylsulfanyl) propenoic acid **[5]**. Using the methodology as set up by Haefliger and Petrizilka[58] and followed by Harwood and co-workers we attempted the synthesis of *3-(Z)-(benzylsulfanyl) propenoic acid.*

3.1.1. Synthesis of *3-(Z)-(benzylsulfanyl) propenoic acid*

In the extensive literature review completed on the syntheses of isothiazolinones, we envisaged the starting point in the synthesis to be the *3-(Z)-(benzylsulfanyl)propenoic acid* **[5]** prepared by Haefliger and Petrizilka. In this synthesis, propynoic acid **[3]** and toluene-α-thiol **[4]** were refluxed in an ethanolic sodium carbonate solution to yield the product in an 74% yield **(Scheme 1)**.[59]

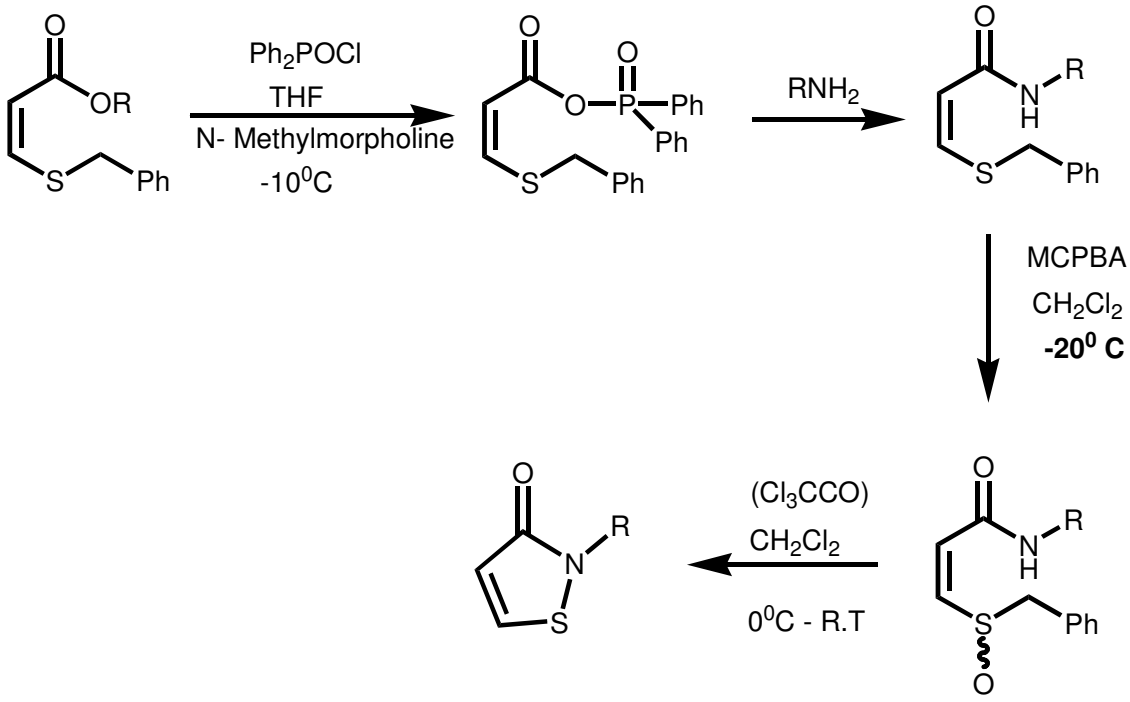


3 **4** **5**

Scheme 1

The addition of thiols to alkynes furnishes preferentially *Z*-adducts under base catalyzed conditions, whilst the non-equivalent non-catalyzed system furnishes *E*-adducts. [60, 61, 62] We were particularly interested in the *Z*-adducts so as to form a range of amides that would eliminate any by-products. Success on the synthesis of 3-(*Z*)-(benzylsulfanyl) propenoic acid was confirmed by the presence of a broad singlet representing the carboxylic acid proton signal in the ^{1}H NMR spectrum at 9.50. Distinct doublets were present at 7.21 and 5.84 representative of the vinylic protons of the reaction product between the alkyne and the thiol. The multiplet representing the aromatic protons was present at 7.28 and also served as confirmation of the formation of *3-(Z)-(benzylsulfanyl) propenoic acid*.

From this point on, we then planned to synthesize the isothiazolinones from the *(Z)-3-(benzylsulfanyl)propenoic acid* following the procedure of Harwood and co-workers.[56] Our proposed synthesis is described in the scheme 2 below:

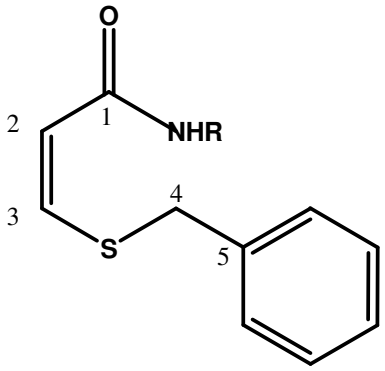


Scheme 2

The five step reaction towards the synthesis of the isothiazolinone derivatives began with *(Z)-3-(benzylsulfanyl)propenoic acid*. This was then treated with diphenylphosphinic chloride and *N*-methylmorpholine in THF at 10°C to furnish the phosphinic ester. This was however not isolated but instead allowed to react directly with a range of amines to produce the corresponding amides. We chose both aliphatic and aromatic amines.

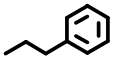
The respective amides were then treated with 3-chloroperbenzoic acid in dichloromethane at -20°C. Successful conversion of the amides into the corresponding sulfoxides was achieved in relatively good yields. The trichloroacetic acid mediated ring closure of the respective sulfoxides was attempted and though successful, the product was present in very low yields.

3.1.2. Syntheses of Amides



R

a  *(Z)-3-(Benzylsulfanyl)-N-butylpropenamide*

b  (*Z)-3-(Benzylsulfanyl)-N-benzylpropenamide*

c  *(Z)-3-(Benzylsulfanyl)-N-(2-ethylphenyl)propenamide*

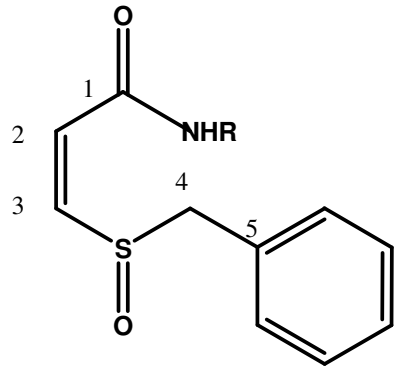
Amide	Percentage Yield
(Z)-3-(Benzylsulfanyl)-N-butylpropenamide	76%
(*Z)-3-(Benzylsulfanyl)-N-benzylpropenamide*	85%
(Z)-3-(Benzylsulfanyl)-N-(2-ethylphenyl)propenamide	88%

Each of the attempted amides above was received in relatively good yields. The proton NMR of *(Z)-3-(benzylsulfanyl)-N-butylpropenamide* revealed doublets at δ 6.56 and 6.26 due to the presence of the vinylic protons. *(Z)-3-(Benzylsulfanyl)-N-butylpropenamide* was confirmed by presence of multiplets at δ 1.36 and 1.28 and a triplet at 0.925, all characteristic of the reaction with *n*-butyl amine.

(*Z)-3-(Benzylsulfanyl)-N-benzylpropenamide* represented vinylic protons at δ 6.79 and 5.60. The presence of two multiplets at δ 7.32-7.22 and 7.22-7.15, representing the two sets of aromatic protons was indicative of the success of the reaction with 2-phenyl-ethylamine. The expected quartet at δ 3.57 and the expected triplet at δ 2.83 were both present in the proton NMR spectrum. The reaction between *3-(Z)-(benzylsulfanyl) propenoic acid* and 2-phenyl-ethylamine was therefore confirmed.

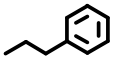
(Z)-3-(Benzylsulfanyl)-N-(2-ethylphenyl)propenamide showed the presence of vinylic protons at δ 6.84 and 5.74. The presence of the broad singlet at 7.29 represented the amide. Multiplets expected at δ 7.33-7.27 and δ 7.27-7.25 were representative of the aromatic protons. A doublet was present at δ 4.46 and δ 3.98, representative of the amide formation of benzyl amine. The reaction was therefore successful under the conditions with a yield of 88%.

3.1.3. Syntheses of the sulfoxides



R

a  *(Z)-3-Benzylsulfinyl)-N-butylpropenamide*

b  *(Z)-3-(Benzylsulfinyl)-N-benzylpropenamide*

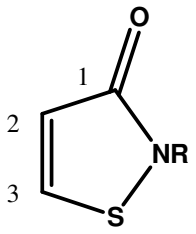
c  *(Z)-3-(Benzylsulfinyl)-N-(2-ethylphenyl)propenamide*

Sulfoxide	Percentage Yield
(Z)-3-Benzylsulfinyl)-N-butylpropenamide	71%
(Z)-3-(Benzylsulfinyl)-N-benzylpropenamide	79%
(Z)-3-(Benzylsulfinyl)-N-(2-ethylphenyl)propenamide	73%

Synthesis of the corresponding sulfoxides of the selected amides in the presence of 3-chloroperbenzoic acid furnished the products in good yields. The common feature present in the proton NMR in the identification of the sulfoxide was the quartet characteristic of the diastereotopic benzylic methylene protons α to the sulfoxide. Quartets for *(Z)-3-Benzylsulfinyl)-N-butylpropenamide, (Z)-3-(benzylsulfinyl)-N-benzylpropenamide* and *(Z)-3-(Benzylsulfinyl)-N-(2-*

ethylphenyl)propenamide were present at δ 4.11, 4.28 and 4.18 respectively. The presence of the quartets confirms the diastereotopic nature of the benzylic methylene protons α to the sulfoxide.

3.1.4. Syntheses of Isothiazolinones



R

a *N-Butylisothiazol-3(2H)-one*

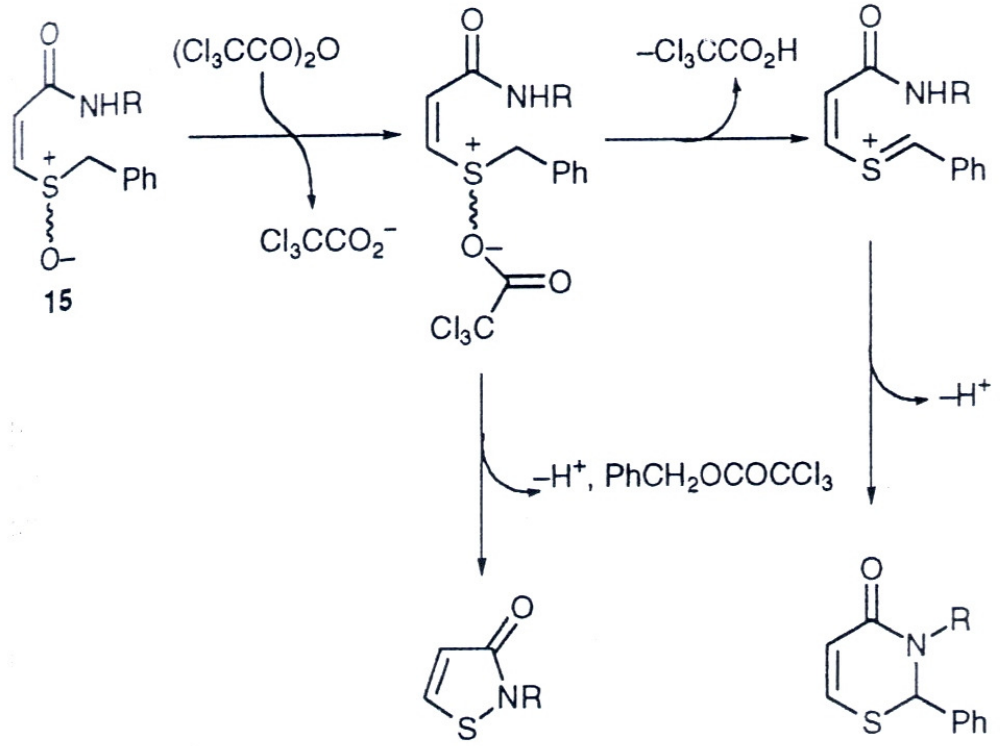
b *N-Benzylisothiazol-3(2H)-one*

c *N-(2-Ethylphenyl)isothiazol-3(2H)-one*

Isothiazolinone	Percentage Yield
N-(2-Ethylphenyl)isothiazol-3(2H)-one	56%

Of the selected isothiazolinones, success was achieved only with *N-(2-ethylphenyl)isothiazol-3(2H)-one* in a yield of 56%. Attempts to produce *N-butylisothiazol-3(2H)-one* and *N-benzylisothiazol-3(2H)-one* were unsuccessful. This could be attributed to one of two reasons. One would be the presence of

electron deficit substituents attached to the nitrogen and the other would be the mechanism behind the Pummerer rearrangement as discussed below.

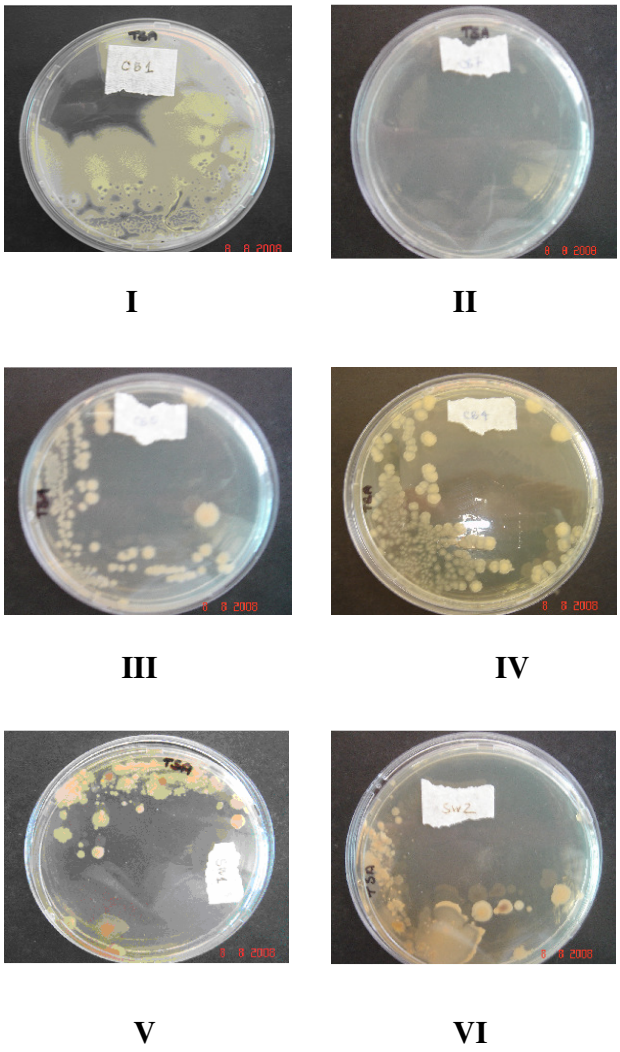


Scheme 3

Scheme 3 above represents the Pummerer rearrangement that explains the mechanism behind possible by-products that may be produced during the trichloroacetic anhydride mediated ring closure of the sulfoxides. The Pummerer rearrangement describes the electrophillic attack of trichloroacetic anhydride on the sulfonium species and that, with the elimination of trichloroacetic acid, forms the six-membered ring closure as opposed to the required five-remembered ring closure. Significant decreases in yields during this reaction can be attributed to the Pummerer rearrangement.

3.2. Part B- Bacterial Culture Growth

Cultures isolated from previous biodegradation experiments were successfully revived using tryptone soy broth and incubated at 30°C on a shaker for 24 hours. Figure 17 below shows the bacterial colonies present when plated on tryptone soy agar plates that were previously prepared. The control showed no growth at all as expected.



I **II**

III **IV**

V **VI**

Figure 17: Images of Agar plates of **I**- *B. subtilis*; **II** – Control; **III** – *Citrobacter freundii*; **IV** – *B. pumilus*; **V** – Sewage Consortium 1; **VI** - Sewage Consortium 2

Colour changes were observed in each of the Erlenmeyer flasks in which microbial growth was induced. Figure 18 below represents the control and each of the sewage consortiums that were inoculated into the bacterial growth medium (tryptone soy broth) and incubated at 30°C on a shaker for 24 hours.

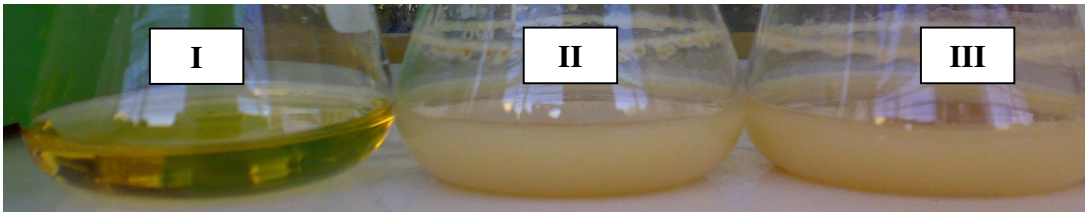


Figure 18: Colour changes observed in bacterial growth medium. I – Control Flask; II and III - induced growth of sewage consortiums.

Although the greatest differences in colour were observed in each of the sewage consortiums giving a milky appearance as compared to the control flask, differences were also observed between the gram negative and gram positive strains that were inoculated for revival. Figure 19 below is a visual representation of the colour changes.

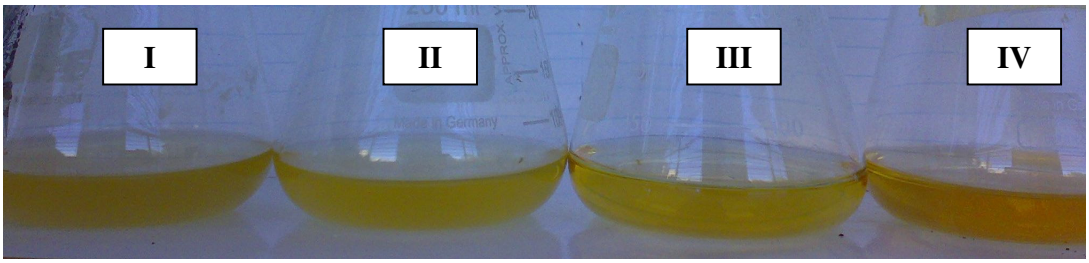


Figure 19: Colour changes observed in bacterial growth medium (tryptone soy broth) of two Gram positive (I- *B. subtilis*, II- *B. pumilus*) and two Gram negative strains (III- *P. aeruginosa*, IV- *Citrobacter freundii*)

3.3. Part C- Toxicity Test

Bacterial growth in the presence of diazolidinyl urea, imidazolidinyl urea and formaldehyde was monitored over a period of 50 days. Positive absorbance values are indicative of bacterial growth within the medium when compared to the control. This method measures turbidity and is an indication of the biomass present within a suspension and therefore serves as a measure for bacterial concentration.

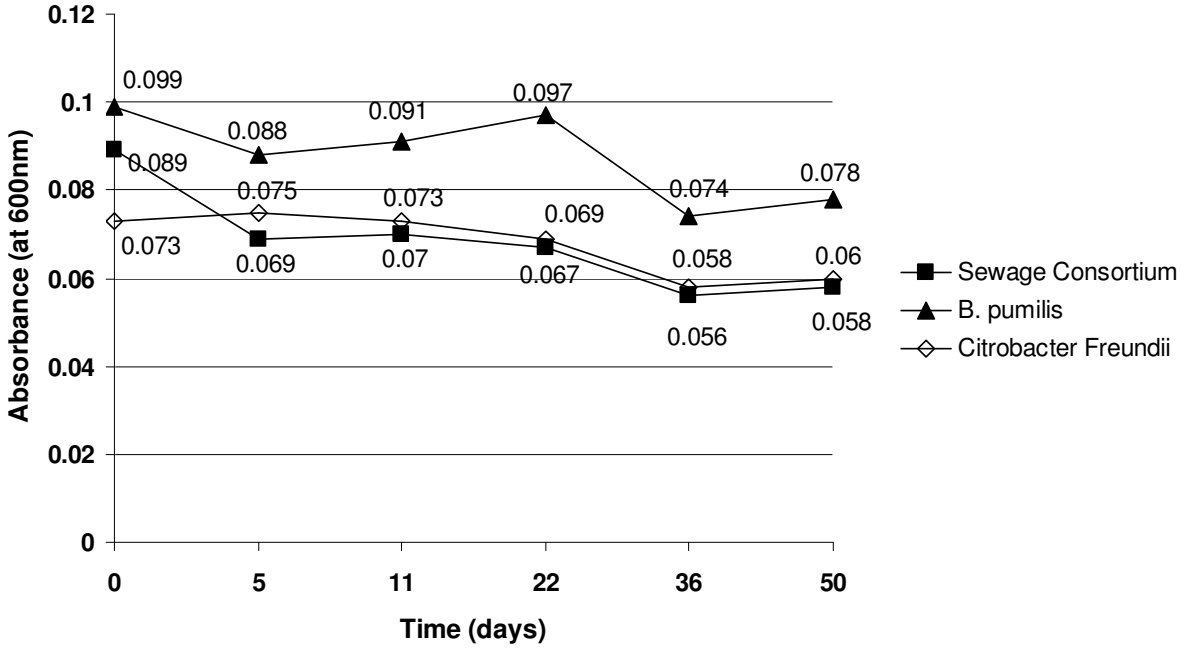


Figure 20: Growth curve of bacteria over 50 days at an absorbance of 600 nm in the presence of diazolidinyl urea

Figure 20 represents the growth pattern of gram positive bacteria (*B. pumilus*), gram negative bacteria (*Citrobacter freundii*) and the sewage consortium in the presence of diazolidinyl urea over a period of 50 days. Day 0 represented initial inoculation of the experimental flasks with each of the chosen microbes and

showed an increase in absorbance thus confirming the presence of bacteria. Gram negative bacteria (*Citrobacter freundii*) showed the most resistance to diazolidinyl urea with a decrease in growth of 17.8% whereas gram positive bacteria (*B. pumilus*) showed a decrease in growth of 21.2%. The sewage consortium showed an unexpected decrease in growth of 34.8%. Diazolidinyl urea was therefore effective in reducing bacterial growth but however not effective in completely eliminating bacteria.

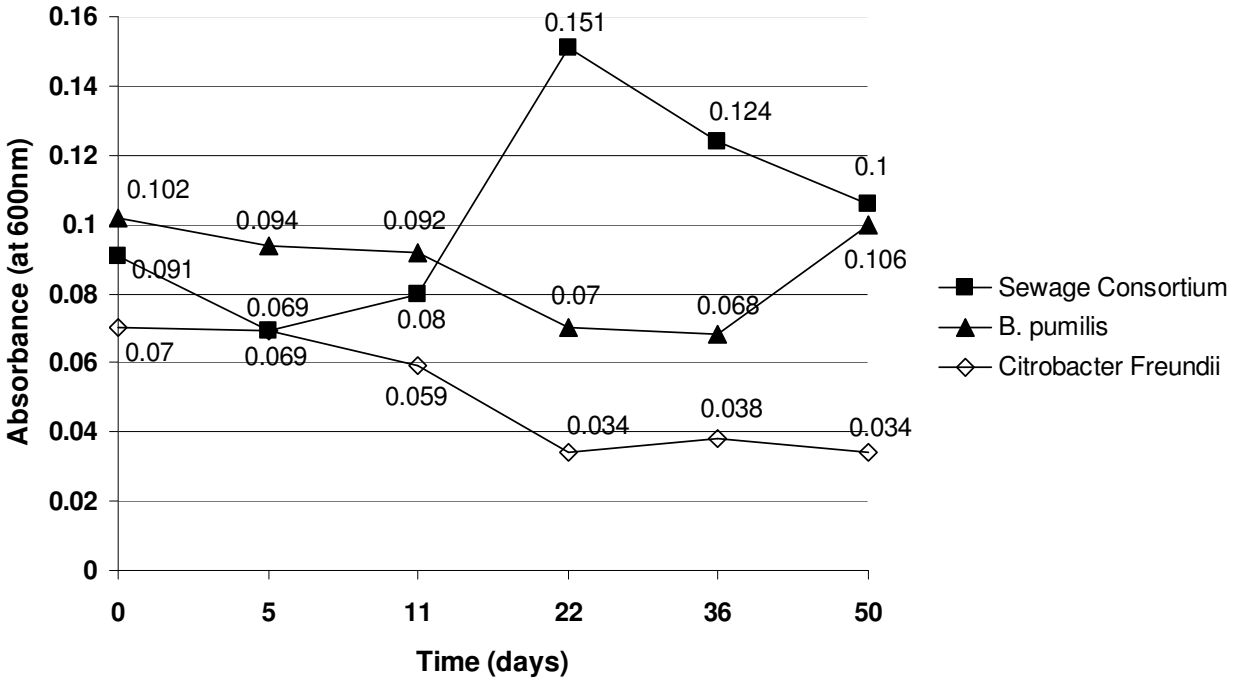


Figure 21: Growth curve of bacteria over 50 days at an absorbance of 600 nm in the presence of imidazolidinyl urea

Figure 21 represents the growth pattern of gram positive bacteria (*B. pumilus*), gram negative bacteria (*Citrobacter freundii*) and the sewage consortium in the presence of imidazolidinyl urea over a period of 50 days. The sewage consortium

showed an increase in growth of 16.5% and therefore exhibited the most resistance to the imidazolidinyl urea. A spike in sewage consortium growth at day 22 was representative of the exponential growth phase in a typical microbial growth curve within a closed system. Growth of 65.9% was observed in the exponential phase. Gram negative bacteria (*Citrobacter freundii*) showed a decrease in growth of 51.4% whereas gram positive bacteria (*B. pumilus*) showed a decrease in growth of 1.96%. Gram negative bacteria (*Citrobacter freundii*) was therefore the most susceptible to imidazoldinyl urea.

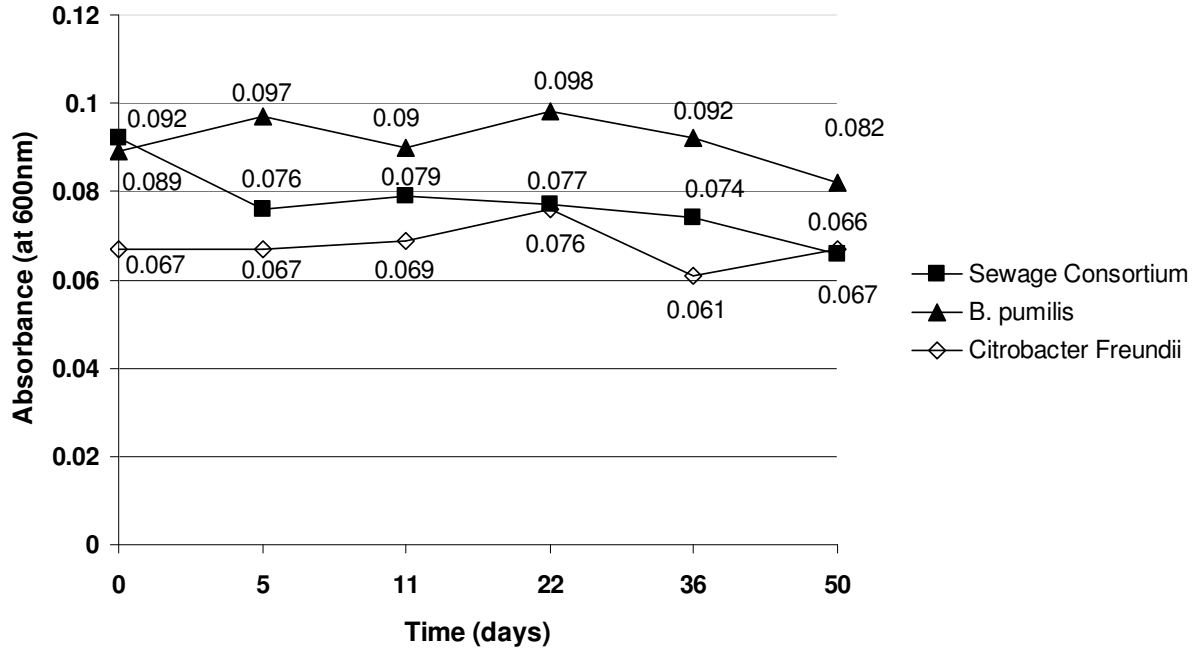


Figure 22: Growth curve of bacteria over 50 days at an absorbance of 600 nm in the presence of formaldehyde

Figure 22 represents the growth pattern of gram positive bacteria (*B. pumilus*), gram negative bacteria (*Citrobacter freundii*) and the sewage consortium in the presence of imidazolidinyl urea over a period of 50 days. The sewage consortium

showed a decrease in growth of 28.3%. Gram negative bacteria (*Citrobacter freundii*) showed no difference in growth and remained in the stationary phase whereas gram positive bacteria (*B. pumilus*) showed a decrease in growth of 7.9%. A spike in gram negative bacteria (*Citrobacter freundii*) growth was observed at day 22 with in increase of 13.4%. This is expected in a typical bacterial growth curve during the exponential phase. The most resistant strain to formaldehyde was therefore gram negative bacteria (*Citrobacter freundii*).

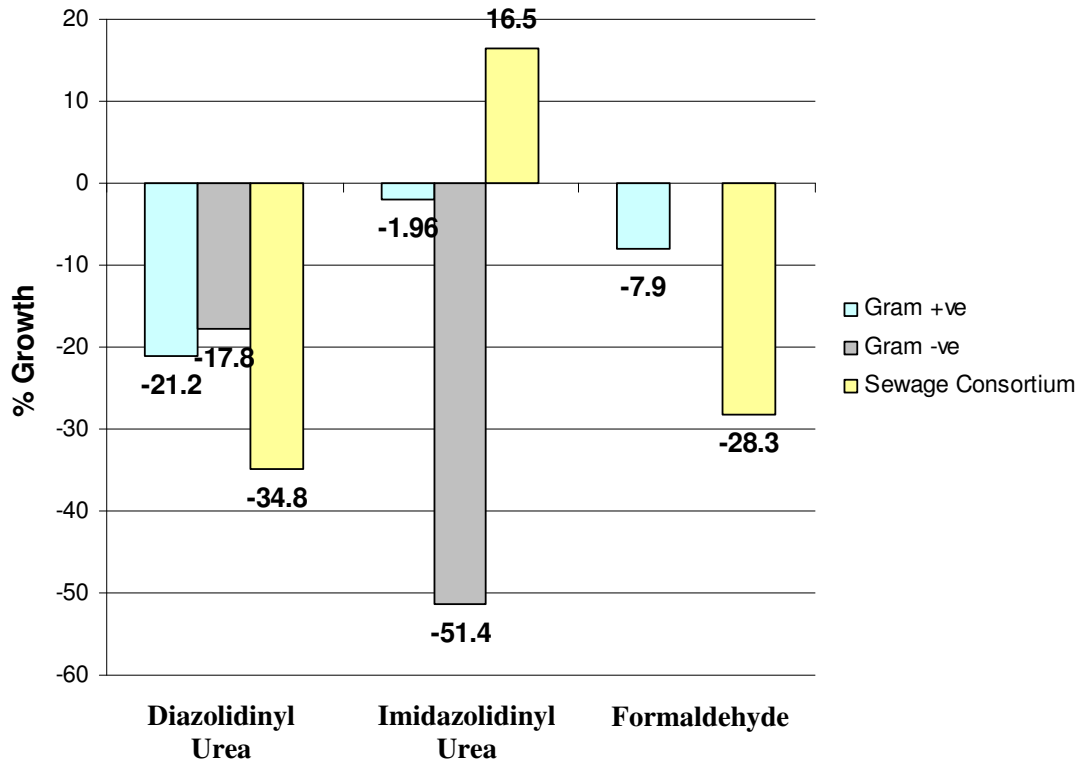


Figure 23: Summary of growth of gram positive bacteria (*B. pumilus*), gram negative bacteria (*Citrobacter freundii)* and the sewage consortium over a period of 50 days

The sewage consortium showed the maximum growth in the presence of imidazolidinyl urea and was therefore ineffective in inhibiting bacterial growth of the sewage consortium. Imidazolidinyl urea was however very effective in reducing growth of both gram positive (*B. pumilus*) and gram negative bacteria (*Citrobacter freundii*). Diazolidinyl urea was effective in reducing growth of gram positive (*B. pumilus*), gram negative (*Citrobacter freundii*) and the sewage consortium. Formaldehyde had no effect on gram negative bacteria (*Citrobacter freundii*) but did effectively reduce growth of both the sewage consortium and gram positive bacteria (*B. pumilus*). The preservative that displayed the best efficacy and resistance to gram positive bacteria, gram negative bacteria as well as the sewage consortium was therefore diazolidinyl urea.

3.4. Part C- Biodegradation assessment of selected industrial preservatives by high performance liquid chromatography (HPLC)

Standards for diazolidinyl urea at concentrations of 50 ppm, 100 ppm, 150 ppm, 200 ppm and 250 ppm were made up. Table 1 below shows the peak areas that were detected using HPLC.

Table 2: Concentration of diazolidinyl urea vs peak area

Concentration (ppm)	Peak Area
50	58.76
100	115.91
150	213.25
200	269.15
250	269.15

Concentration of standards at 50 ppm, 100 ppm, 150 ppm, 200 ppm and 250 ppm was then plotted against peak area on a linear graph.

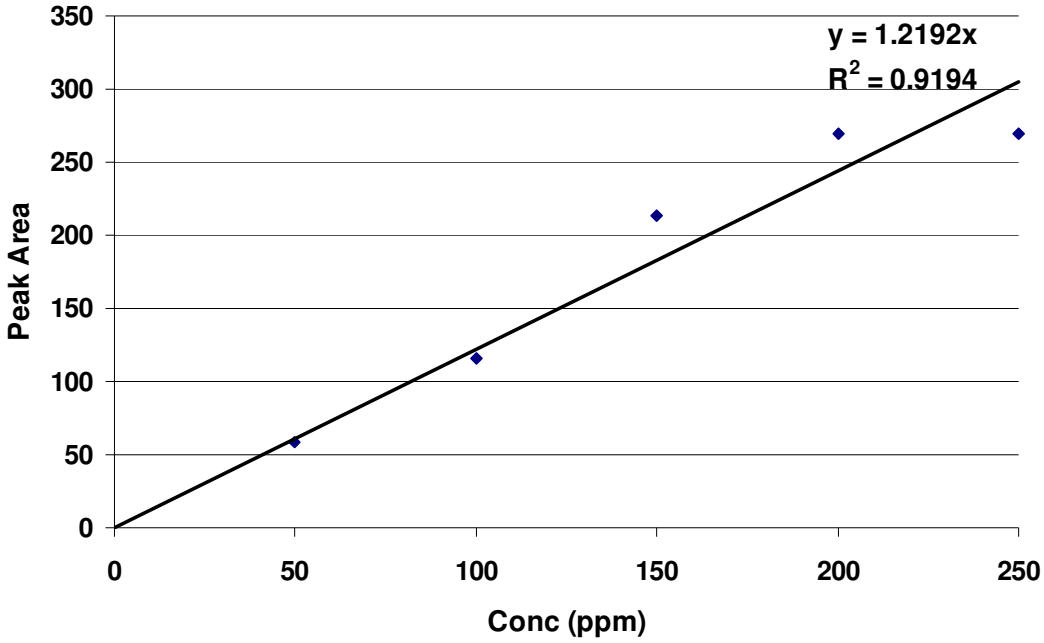


Figure 24: Standard curve of diazolidinyl urea

Table 3: Concentration of diazolidinyl urea after 50 days under experimental conditions

Experimental conditions	Concentration (Day 0)	Concentration (Day 50)	Percentage biodegradation
Gram positive (*B.pumilus*)	266.94	250.01	5.77%
Gram negative (*Citrobacter freundii*)	715.78	343.72	51.40%
Sewage Consortium	348.83	310.07	10.53%

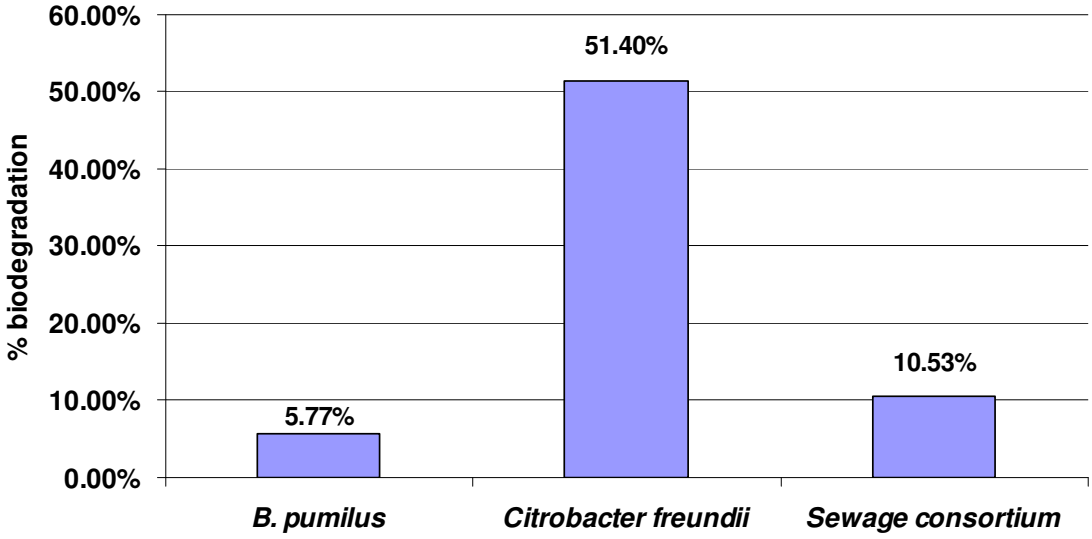


Figure 25: Graphical representation of biodegradation of diazolidinyl urea

Gram positive bacteria (*B. pumilus*) and the sewage consortium biodegraded diazolidinyl urea at 5.77% and 10.53% respectively. The most significant biodegradation however was observed by gram negative bacteria (*Citrobacter freundii*) at 51.40%. Based on the results from the toxicity test, it appears that even though bacterial growth was reduced by the incorporation of diazolidinyl urea into the experimental flasks, the decrease is growth was not significant enough to inhibit effective biodegradation. The sewage consortium served to simulate conditions within the natural environment and biodegradation of 10.53% was observed over a period of 50 days.

Table 4: Concentration of imidazolidinyl urea vs peak area

Concentration (ppm)	Peak Area
50	68.0668
100	106.0684
150	161.5696
200	187.601
250	249.292

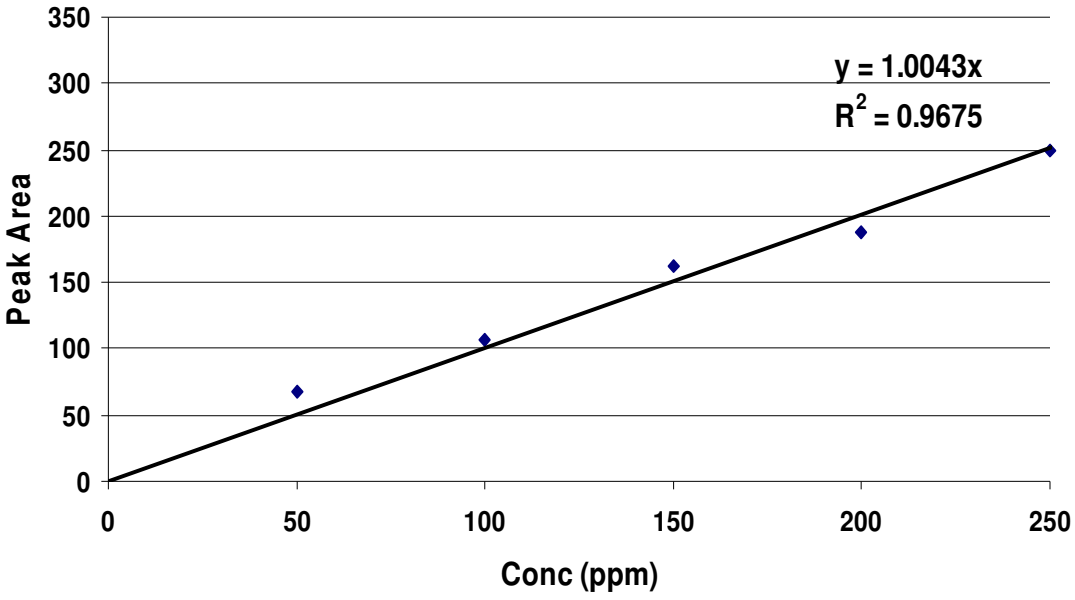


Figure 26: Standard curve of imidazolidinyl urea

Table 5: Concentration of imidazolidinyl urea after 50 days under experimental conditions

Experimental conditions	Concentration (Day 0)	Concentration (Day 50)	Percentage biodegradation
Gram positive (*B.pumilus*)	296.17	272.40	6.32%
Gram negative (*Citrobacter freundii*)	296.17	280.71	3.51%
Sewage Consortium	483.90	431.95	9.03%

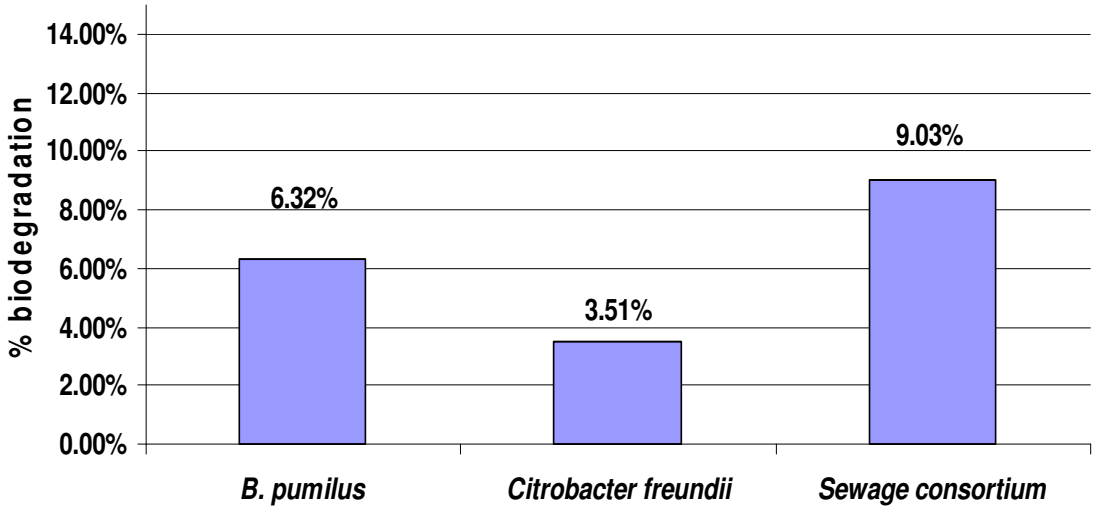


Figure 27: Graphical representation of biodegradation of imidazolidinyl urea

Maximum biodegradation of imidazolidinyl urea was observed by the sewage consortium at a percentage of 9.03%. Gram positive bacteria (*B. pumilus*) and gram negative bacteria (*Citrobacter freundii*) biodegraded imidazolidinyl urea at 6.32% and 3.51% respectively. Based on the results obtained from the toxicity test, the sewage consortium was expected to show the most biodegradation in comparison to the gram positive bacteria (*B. pumilus*) and gram negative bacteria (*Citrobacter freundii*) as it showed a percentage growth of 16.5%. This therefore confirmed that the sewage consortium showed the most resistance to imidazolidinyl urea and therefore the medium provided ideal conditions for biodegradation.

Table 6: Concentration of formaldehyde vs peak area

Concentration (ppm)	Peak Area
50	71.02
100	71.91
150	797.96
200	809.26
250	816.25

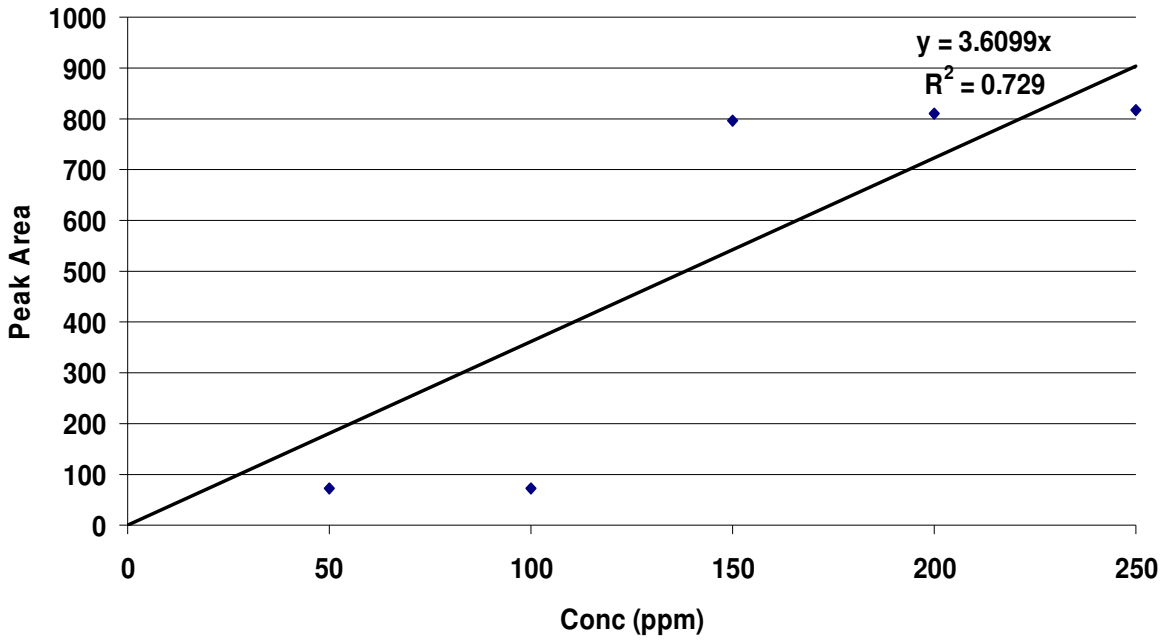


Figure 28: Standard curve of formaldehyde

Standards were created for formaldehyde and peak areas were generated through high performance liquid chromatography. However no results were obtained under the experimental conditions. Formaldehyde could not be detected at all after being subjected to various bacterial strains over period of 50 days. This could be attributed to either the evapouration of formaldehyde over a period of 50 days or the more likely possibility that based on the results from the toxicity test, formaldehyde was already depleted in inhibiting the growth of the sewage consortium and gram positive bacteria (*B. pumilus*). Growth was inhibited by 28.3% and 7.9% respectively. The concentration of formaldehyde used for this study was therefore not sufficient.

CHAPTER 4

CONCLUSIONS

Successful synthesis of *3-(Z)-(benzylsulfanyl) propenoic acid*, the precursor to the five step synthesis of isothiazolinone derivatives was achieved in a yield of 74%. The use of sodium carbonate as the base to catalyze this reaction proved to be successful. The reaction of *3-(Z)-(benzylsulfanyl) propenoic acid* with *n*-butyl amine, 2-phenyl-ethylamine and benzyl amine all successfully yielded the corresponding amides in 76%, 85% and 88% respectively. This confirmed the effectiveness of having produced the phosphinic ester as an intermediate by using diphenylphosphinic chloride and reacting the ester directly with the selected amine. The corresponding sulfoxides were produced in yields of 71%, 79% and 73%. The appearance of the doublet of doublets in all three proton NMR's representing the diastereotopic benzylic methylene protons α to the sulfoxide was a characteristic feature of the sulfoxide formation. The trichloroacetic anhydride mediated ring closures, although successful in producing *N-(2-ethylphenyl)isothiazol-3(2H)-one* in a very low yield was unsuccessful in producing *N-butylisothiazol-3(2H)-one* and *N-benzylisothiazol-3(2H)-one*. Possible reasons could be the Pummerer rearrangement but this could however also be attributed to the presence of the electron deficit substituents. The nature of the cyclisation reaction is the intramolecular nucleophilic attack on the sulfur by electron rich aromatic substituents on the nitrogen. The low yields can be attributed to the electron deficit substituents attached to the nitrogen and thus producing low yields.

The sewage consortium, gram positive bacteria (*B. pumilus*) and gram negative bacteria (*Citrobacter freundii*) were successfully revived in tryptone soy broth which served as the nutrient medium to allow the cultures to grow.

Toxicity test results revealed that in the presence of diazolidinyl urea, the percentage of bacterial growth was reduced in all three strains. Gram positive bacteria (*B. pumilus*) growth was reduced by 21.2% whereas gram negative bacteria (*Citrobacter freundii*) growth was reduced by 17.8%. The sewage consortium showed the least resistance to diazolidinyl urea with a percentage decrease of 34.8%. Gram negative bacteria (*Citrobacter freundii*) showed the most resistance to diazolidinyl urea.

Gram negative bacteria (*Citrobacter freundii*) and gram positive bacteria (*B. pumilus*) both showed a decrease in bacterial growth in the presence of imidazolidinyl urea. Gram negative bacteria (*Citrobacter freundii*) showed a much more significant decrease in growth than gram positive bacteria (*B. pumilus*). Imidazolidinyl urea therefore had a greater effect on gram negative bacteria than gram positive bacteria. The sewage consortium showed an increase of 16.5% bacterial growth in the presence of imidazolidinyl urea over a period of 50 days. This was expected due to the resistant strains that are present in sewage consortiums.

The presence of formaldehyde had no effect on gram negative bacteria (*Citrobacter freundii*) over a period of 50 days. The strain exhibited stationary phase behaviour by showing neither an increase nor a decrease in growth. The sewage consortium was most susceptible to the presence of formaldehyde under the test conditions showing a decrease in growth of 28.3%. Gram positive bacteria (*B. pumilus*) growth was also reduced by 7.9%.

High performance liquid chromatography analysis revealed that over a period of 50 days, gram negative bacteria (*Citrobacter freundii*) was the most effective in biodegrading diazolidinyl urea. The percentage of biodegradation achieved was 51.4% in comparison to gram positive bacteria (*B. pumilus*) and the sewage consortium at 5.7% and 10.5% respectively.

Results differed with regard to imidazolidinyl urea where the sewage consortium showed a percentage biodegradation of 9% followed by gram positive bacteria (*B. pumilus*) at 6.3% and gram negative bacteria (*Citrobacter freundii*) at 3.5%.

It can therefore be concluded that under natural conditions as simulated by the sewage consortium, both diazolidinyl urea and imidazolidinyl urea would be only partially biodegraded. It is also possible that at different times the composition of the sewage consortium may be different in the concentration of gram positive and gram negative bacteria and therefore the percentage of biodegradation would always be different within the natural environment.

Formaldehyde and the produced isothiazolinone compound could not be detected by HPLC. Experimental conditions may not have been favorable to these two compounds due to the low concentrations that they were utilized in.

Very little is known about the biodegradation of preservatives due to the lack of research in this field and not much literature has been published thus prompting future work in this field.

CHAPTER 5

FUTURE WORK

Isothiazolinone derivatives and precursors were synthesized in very low yields and future work would be to refine the experimental methodology to produce substantial yields that would prove to be commercially viable. The synthesis of a wide range of alternate preservatives in significant yields should also be considered as an option using alternate parent substrates to the isothioazolinone backbone. A method for the determination of the biocidal efficacy of synthesized organic compounds should also be developed.

This study focused specifically on the industrial raw materials used in preservation but the potential to explore raw materials across a broad range of industrial categories is of great importance. Regulations are constantly reviewed within various industries and biodegradation of a wide range of raw materials are constantly under review and speculation. Studies like this one could be useful across a wide range of categories and future work would definitely be to open this study up to a wide range of raw material classes.

Further biodegradation studies would involve the synthesis of structural equivalents to naturally produced biosurfactants and assess their breakdown within the natural environment. Methodology refinement for biodegradation studies should also serve as future work to this biodegradation study as very little literature and information is available on analytical research conducted on biodegradation.

Companies producing raw materials that are introduced into the natural environment should assess their waste on a regular basis and determine methods of increasing the biodegradation within their facilities and releasing a more eco-

friendly effluent into the natural waste water system. This would help in not only maintaining the balance of the eco-system but also preventing resistant strains of bacteria from being produced.

CHAPTER 6

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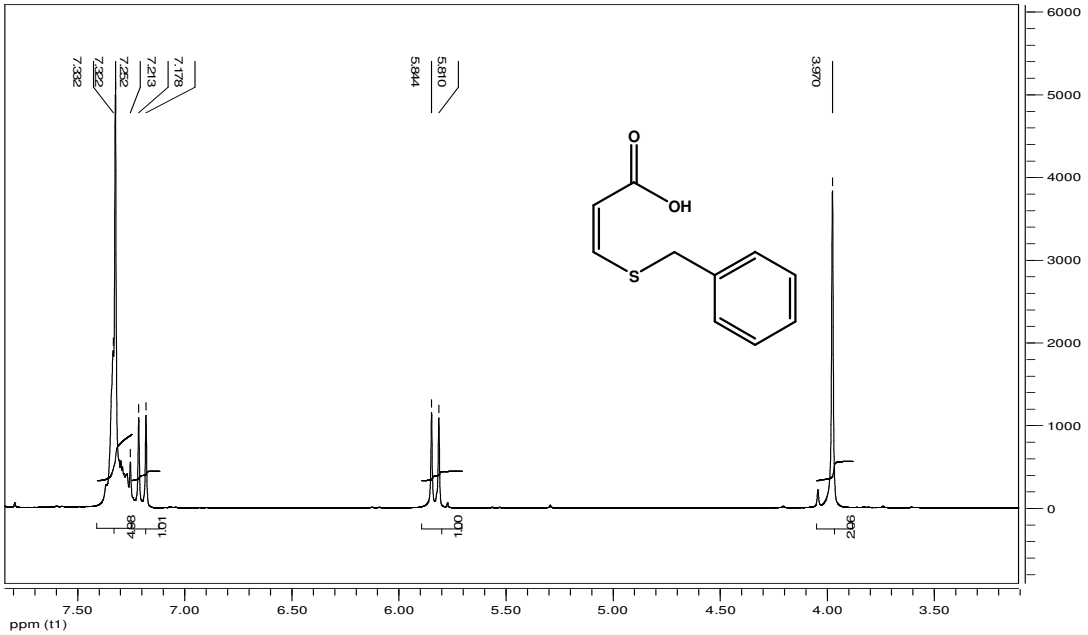
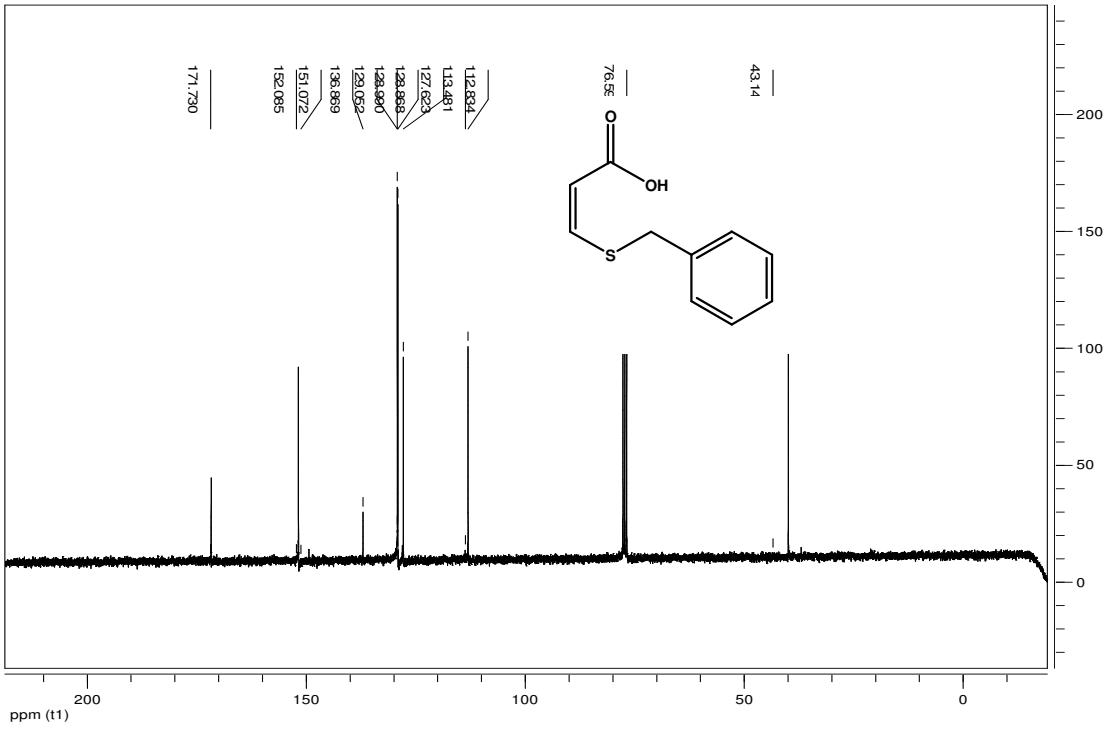
APPENDIX

A1: Selected ^{1}H and ^{13}C NMR Spectra for Part A

A1.1.1 The ^{1}H NMR Spectrum of 3-(*Z*)-(Benzylsulfanyl) propenoic acid



A1.1.2 The ^{13}C NMR Spectrum of 3-(*Z*)-(Benzylsulfanyl) propenoic acid



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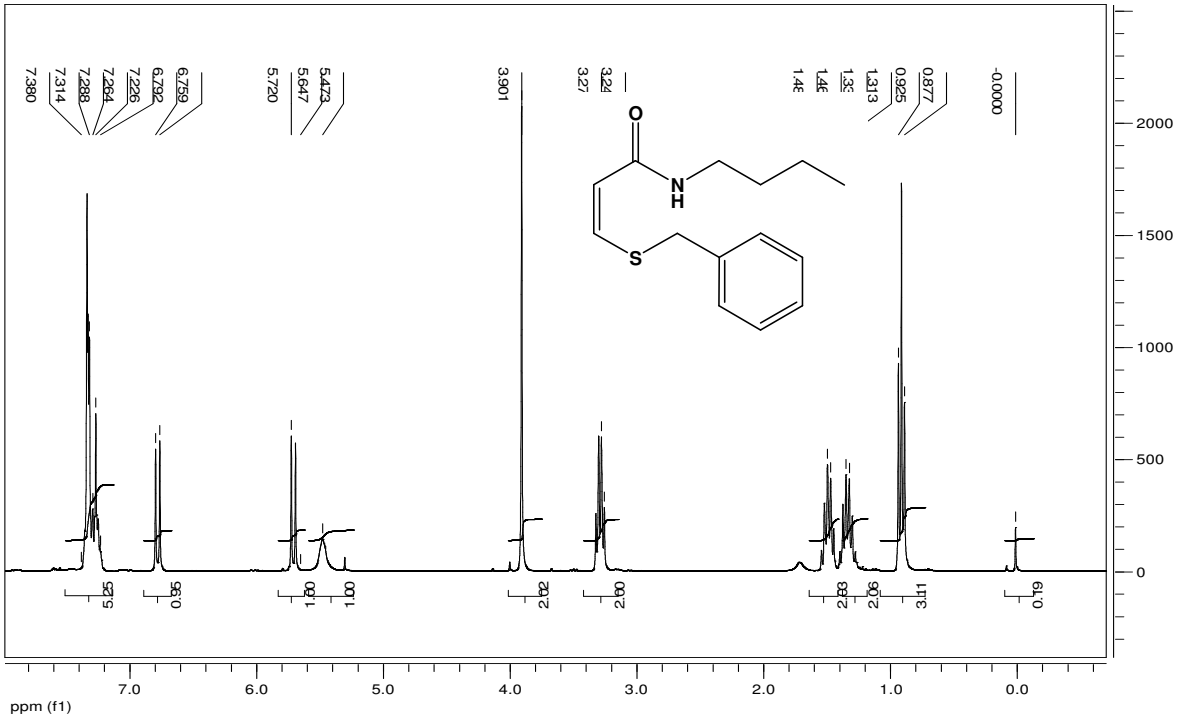
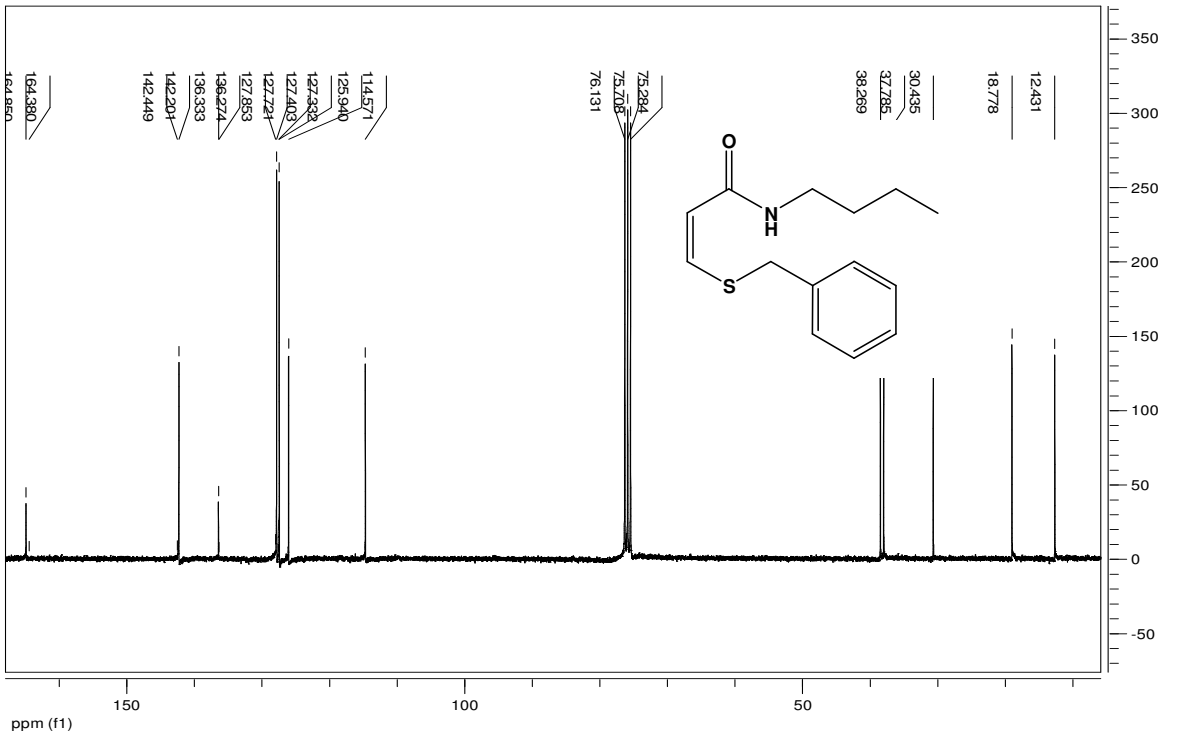
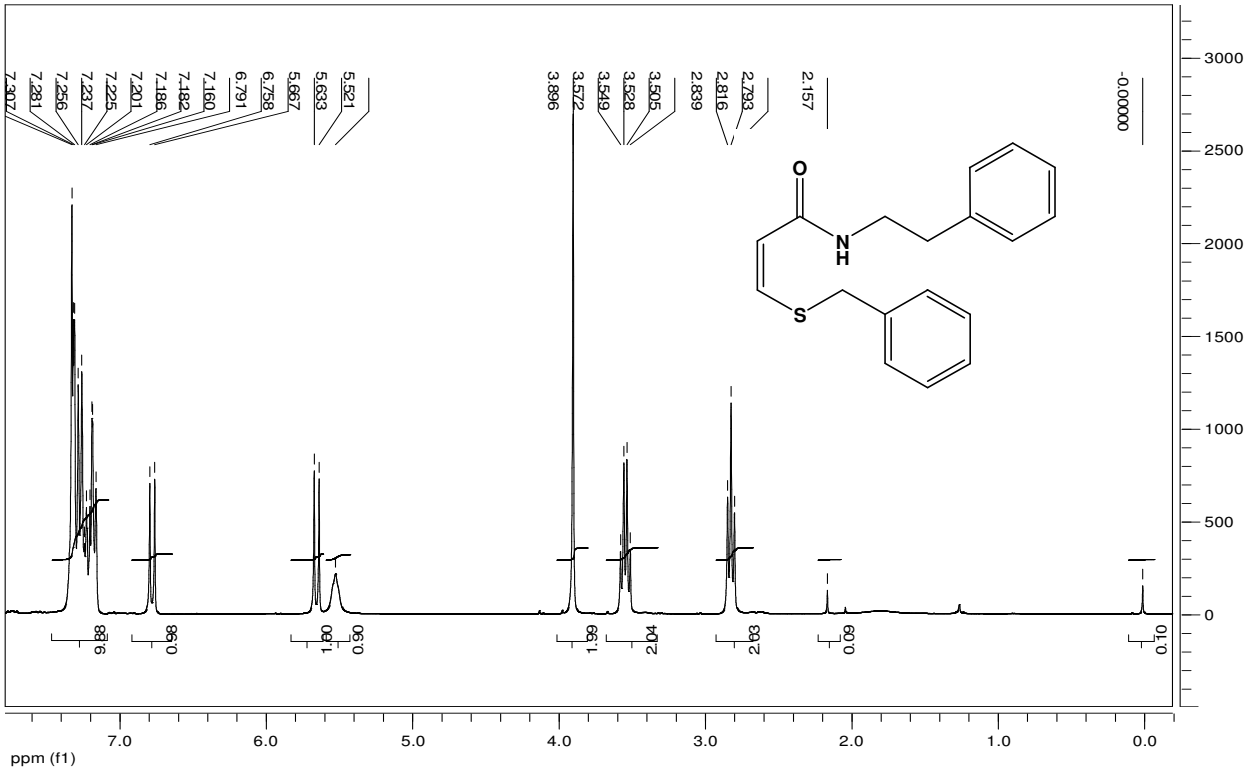


A1.2.2 The ^{13}C NMR Spectrum of *(Z)-3-(Benzylsulfanyl)-N-butylpropenamide*



A1.3.1 The ^{1}H NMR Spectrum of (*Z*)-*3*-*(Benzylsulfanyl)*-*N*-*benzylpropenamide*



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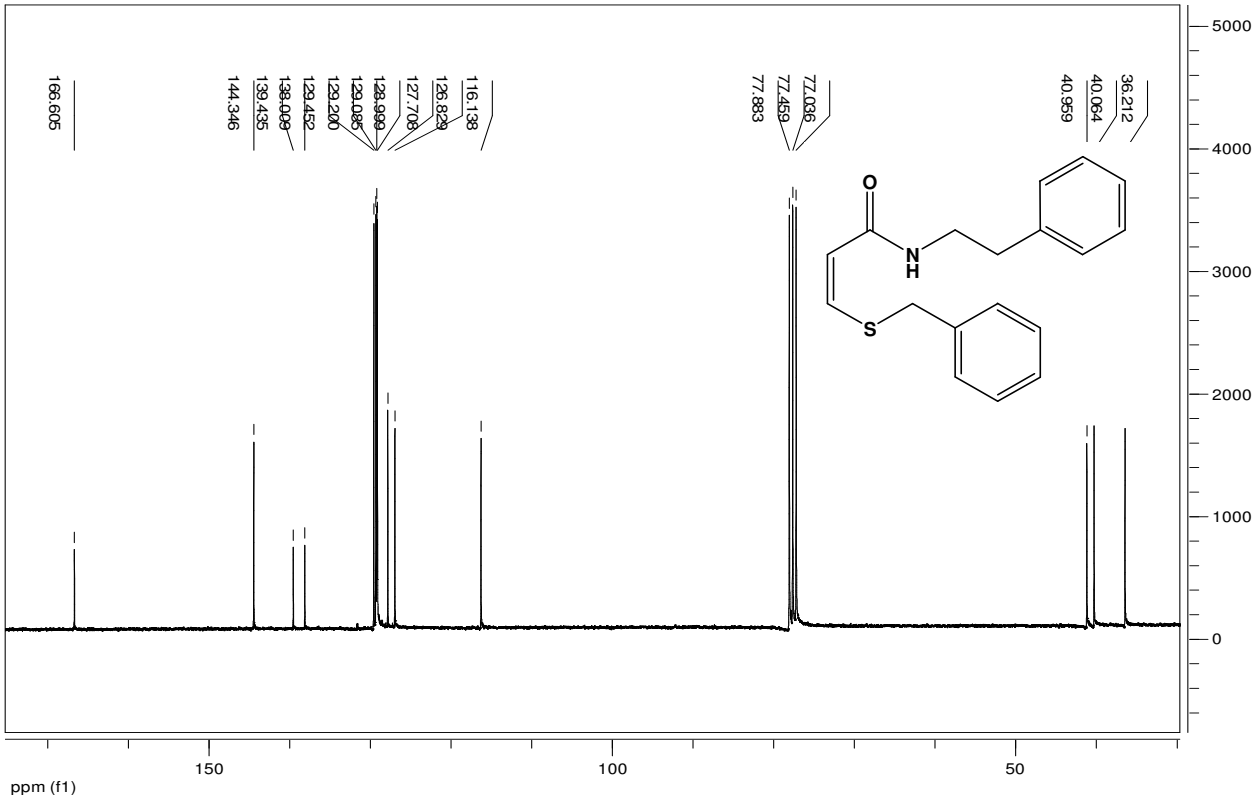

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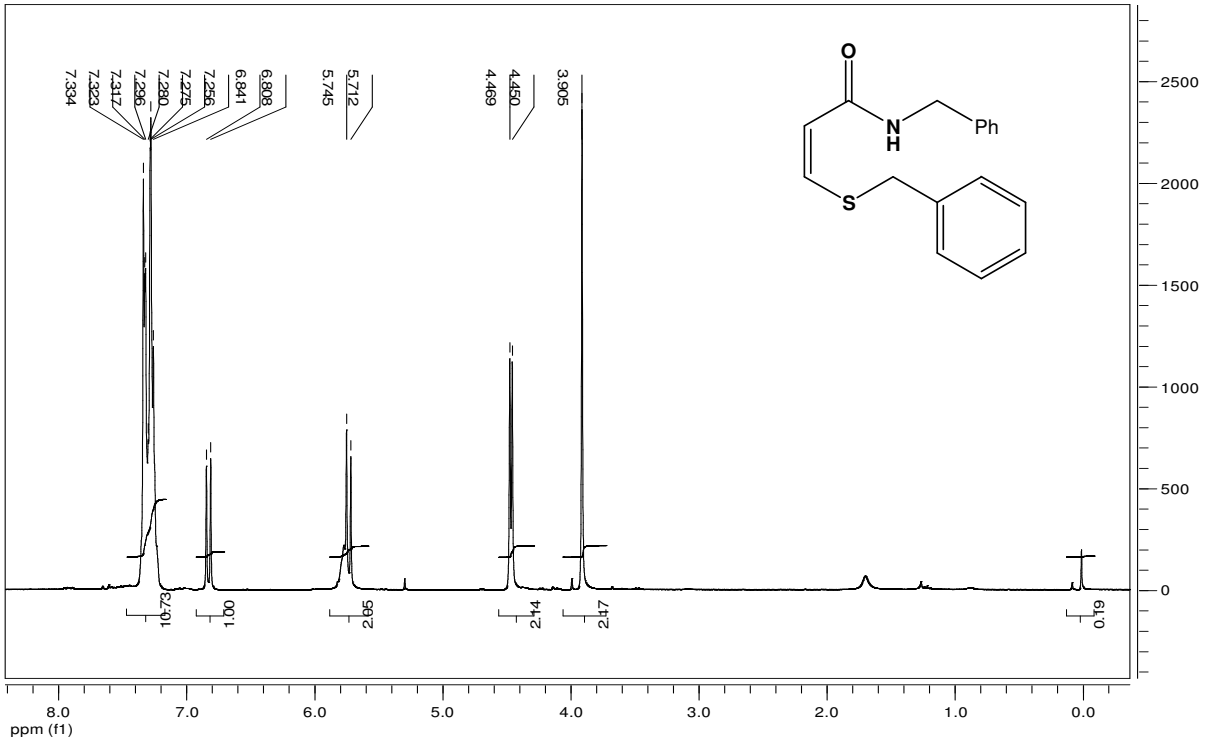
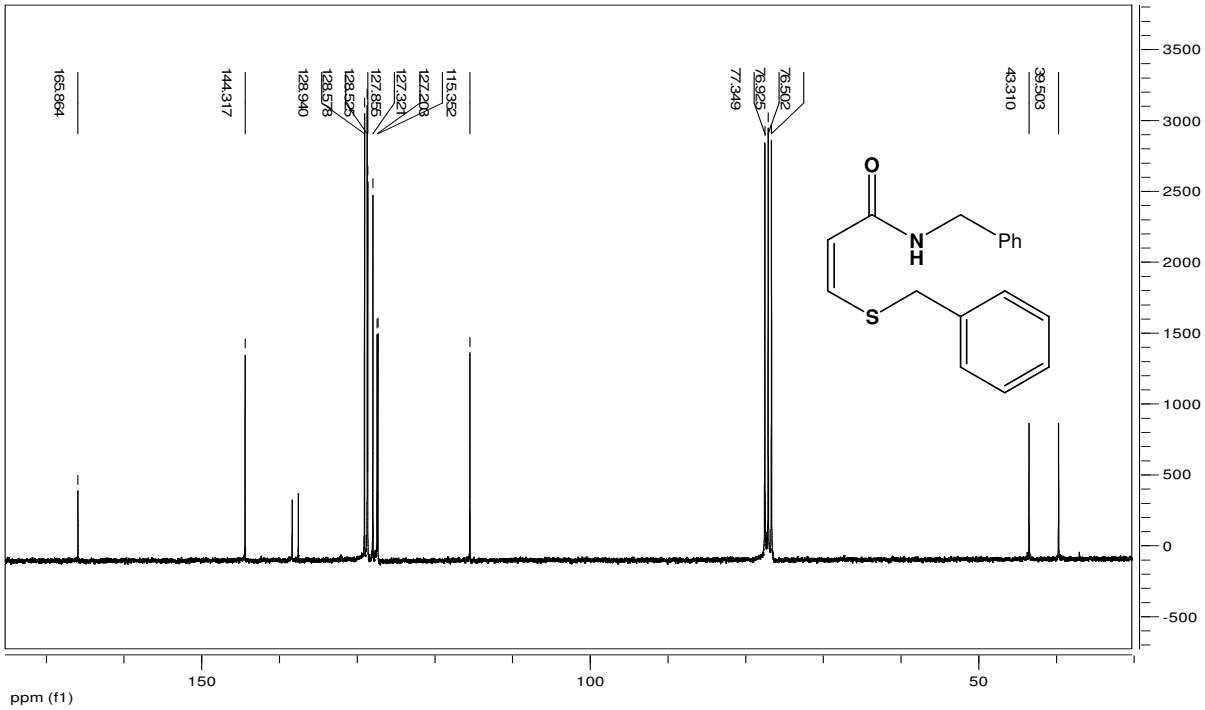


A1.4.2 The ^{13}C NMR Spectrum of *(Z)-3-(Benzylsulfanyl)-N-(2-ethylphenyl)propenamide*



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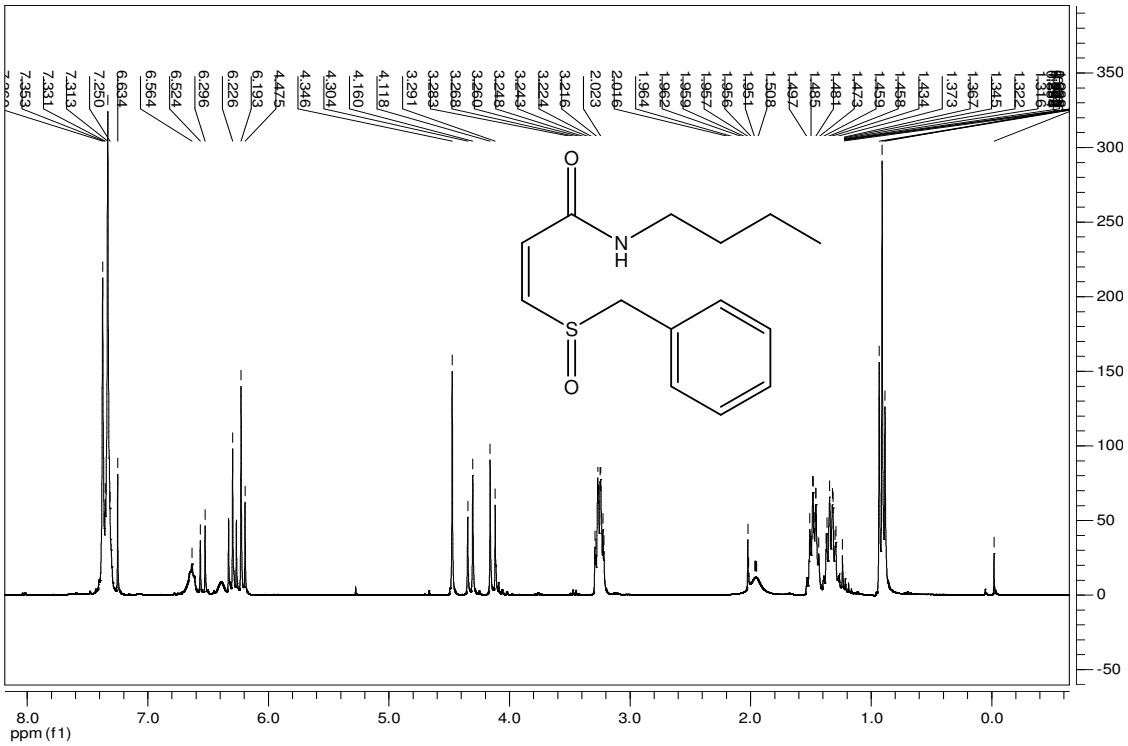

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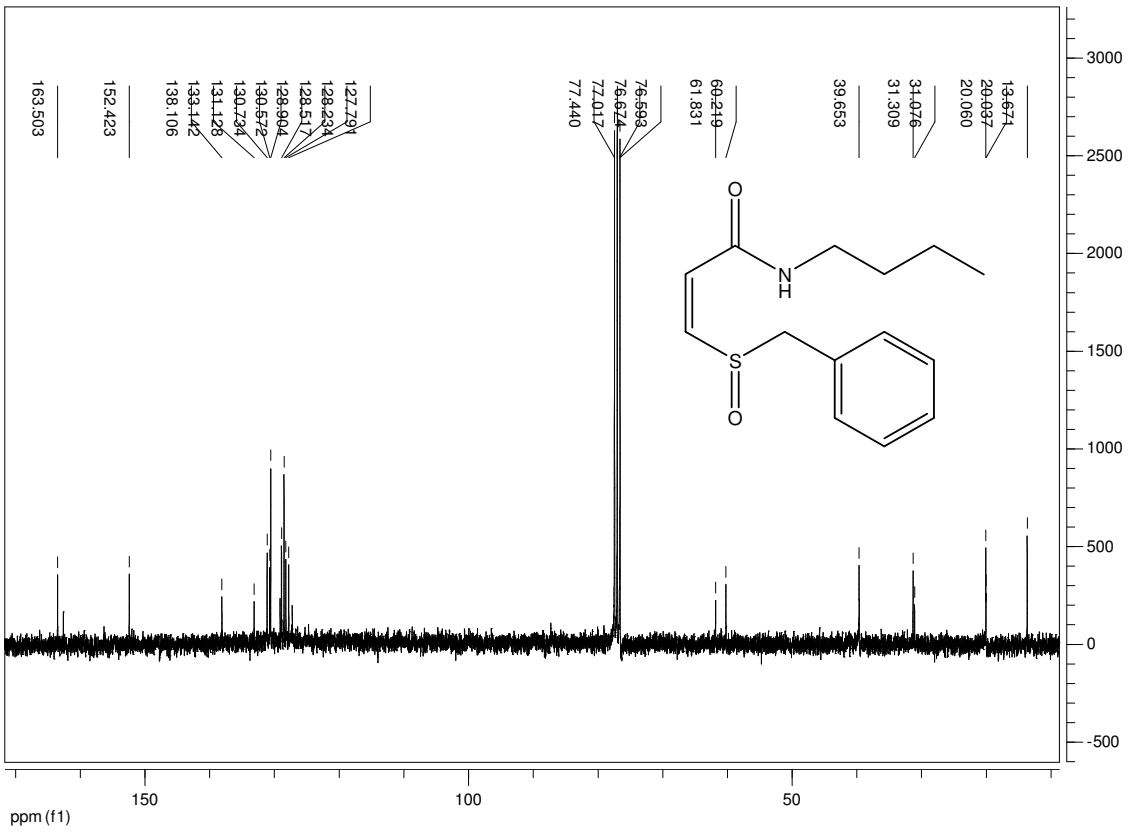
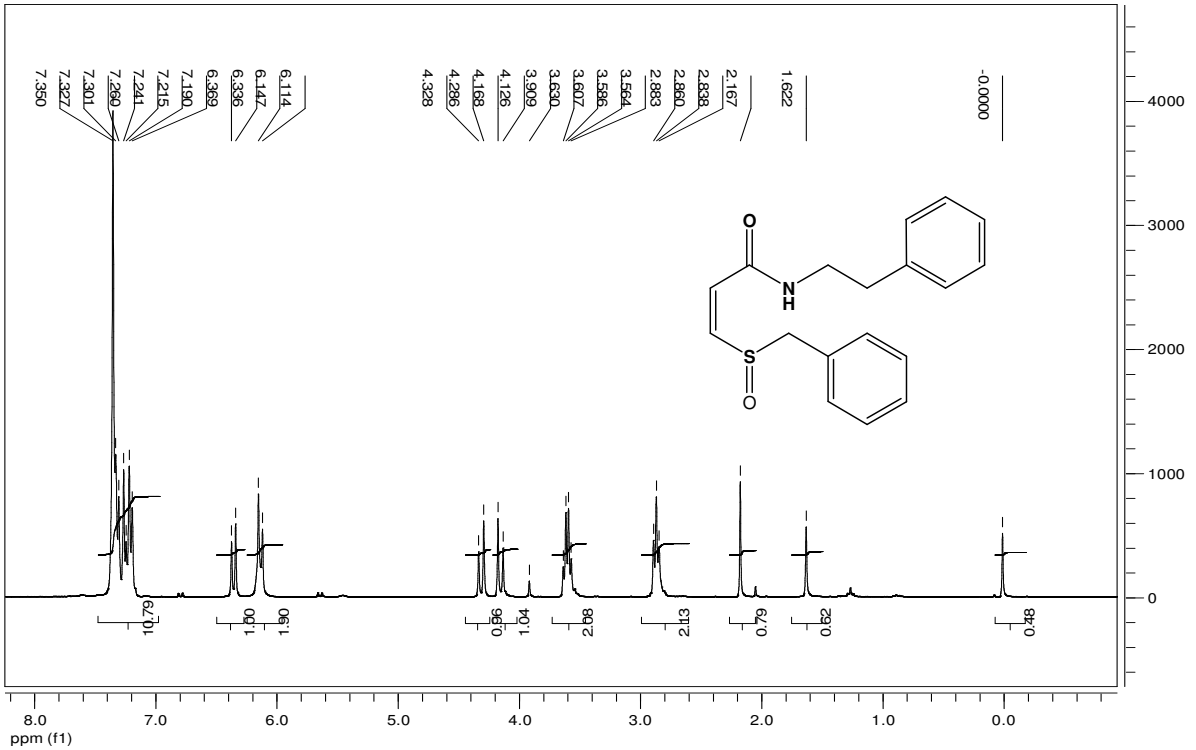


A1.6.1 The ^{1}H NMR Spectrum of *(Z)-3-(Benzylsulfinyl)-N-benzylpropenamide*



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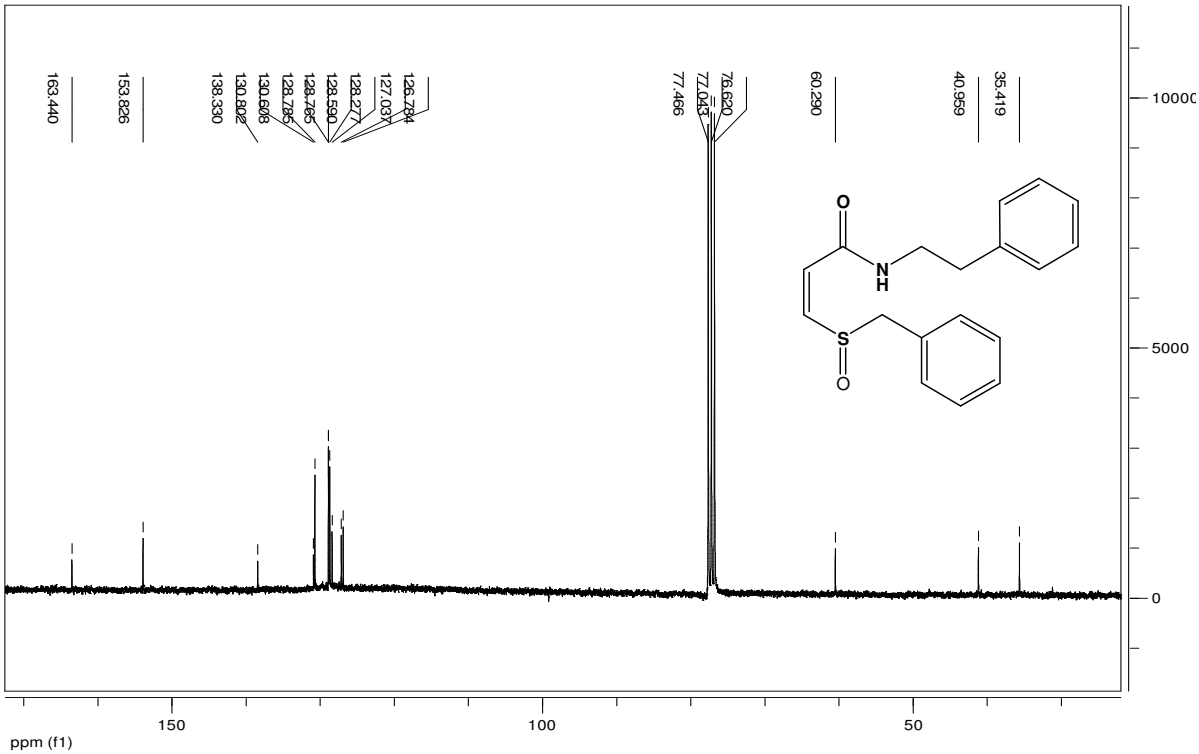
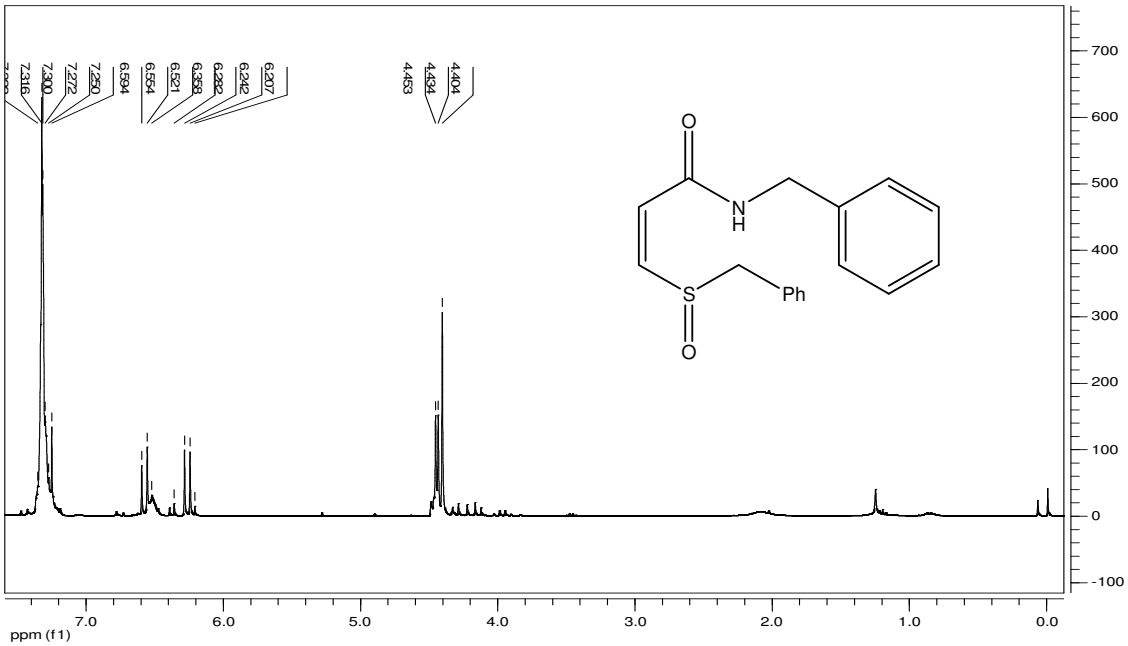


A1.7.1 The ^{1}H NMR Spectrum of *(Z)-3-(Benzylsulfinyl)-N-(2-ethylphenyl)propenamide*



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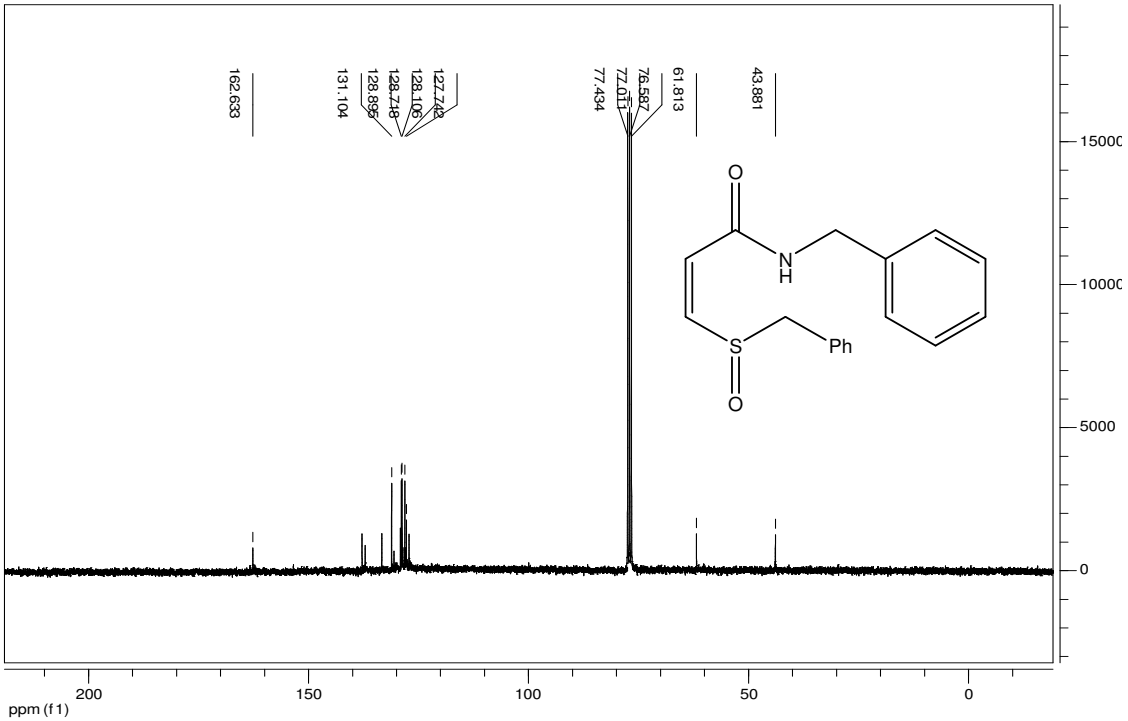

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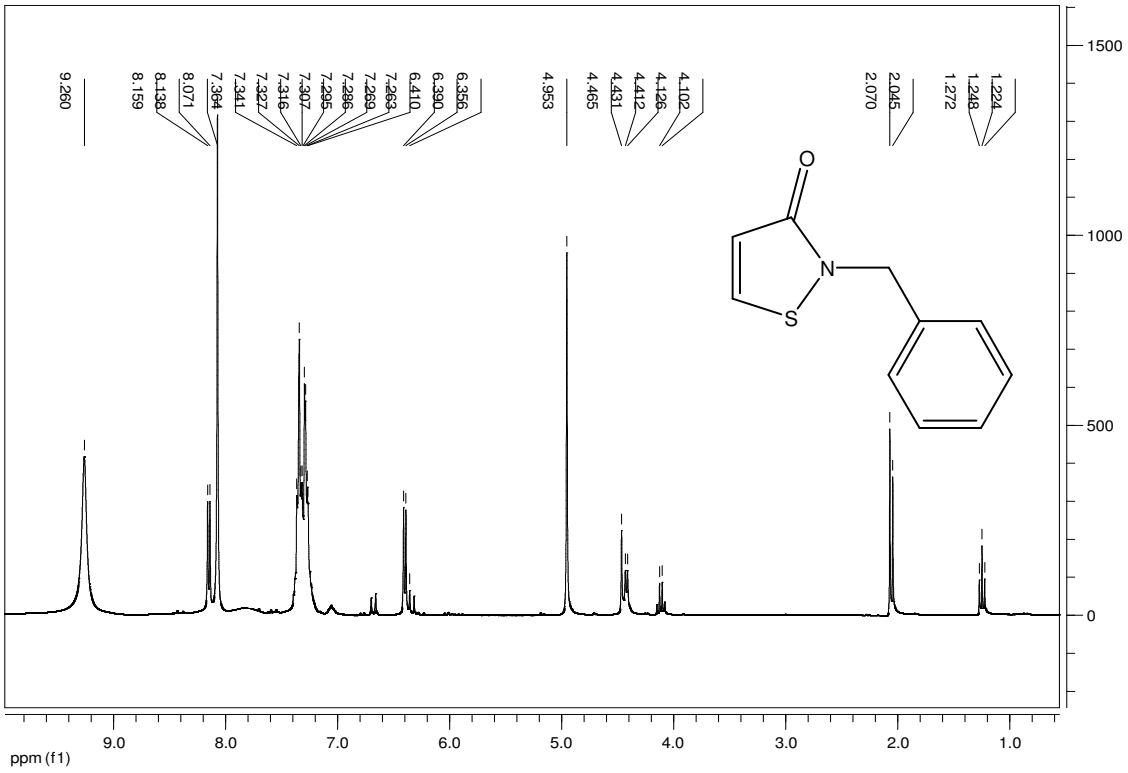

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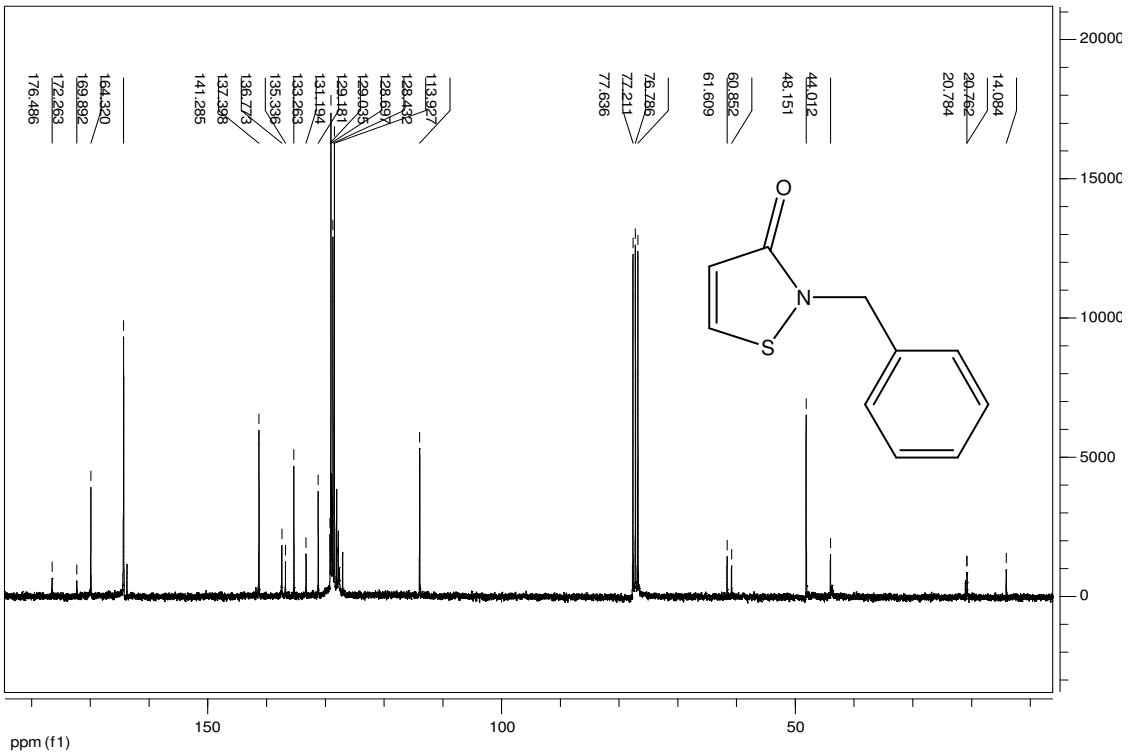

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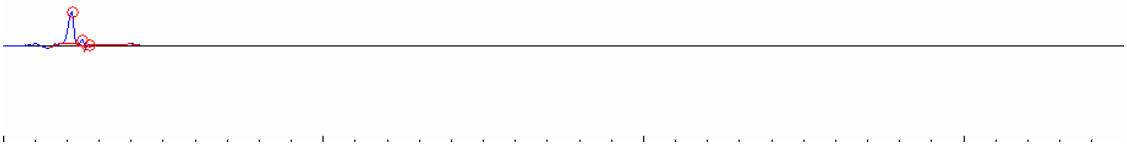
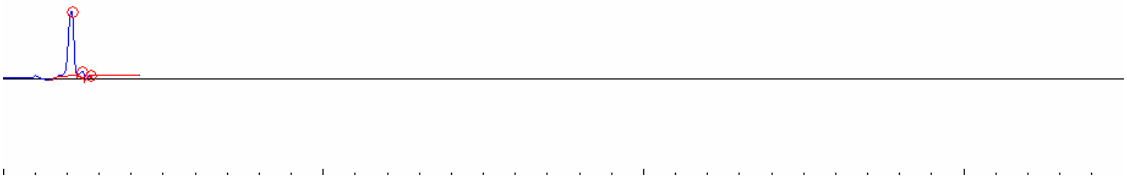


DZ 50 ppm



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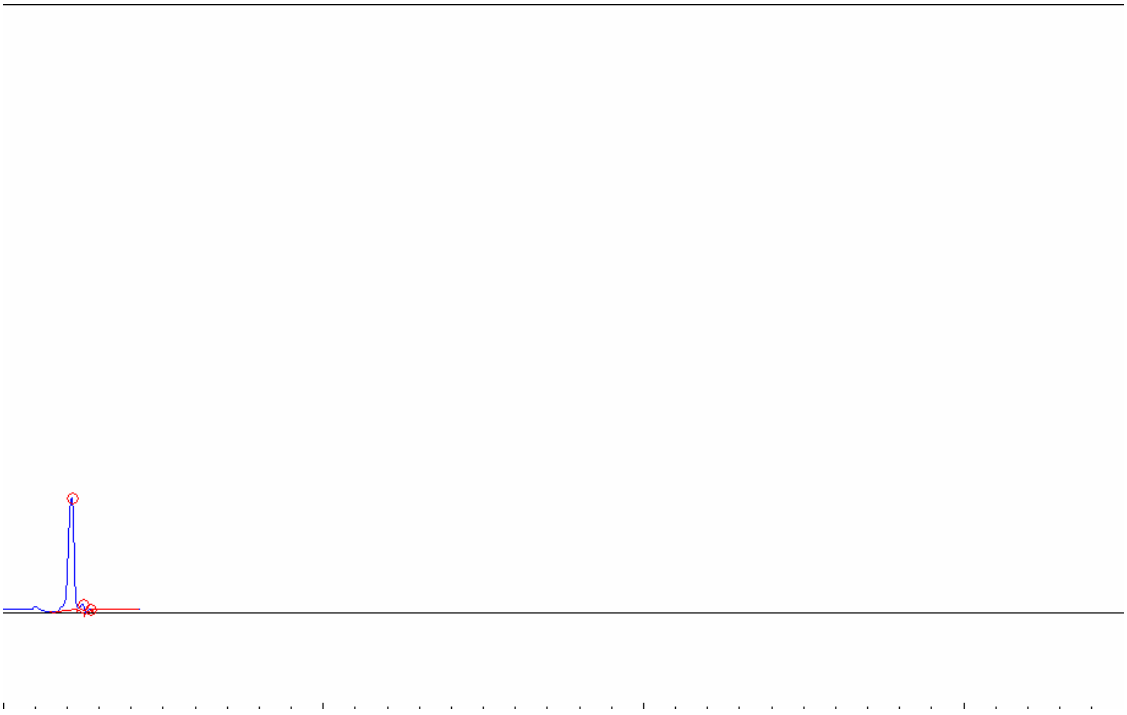
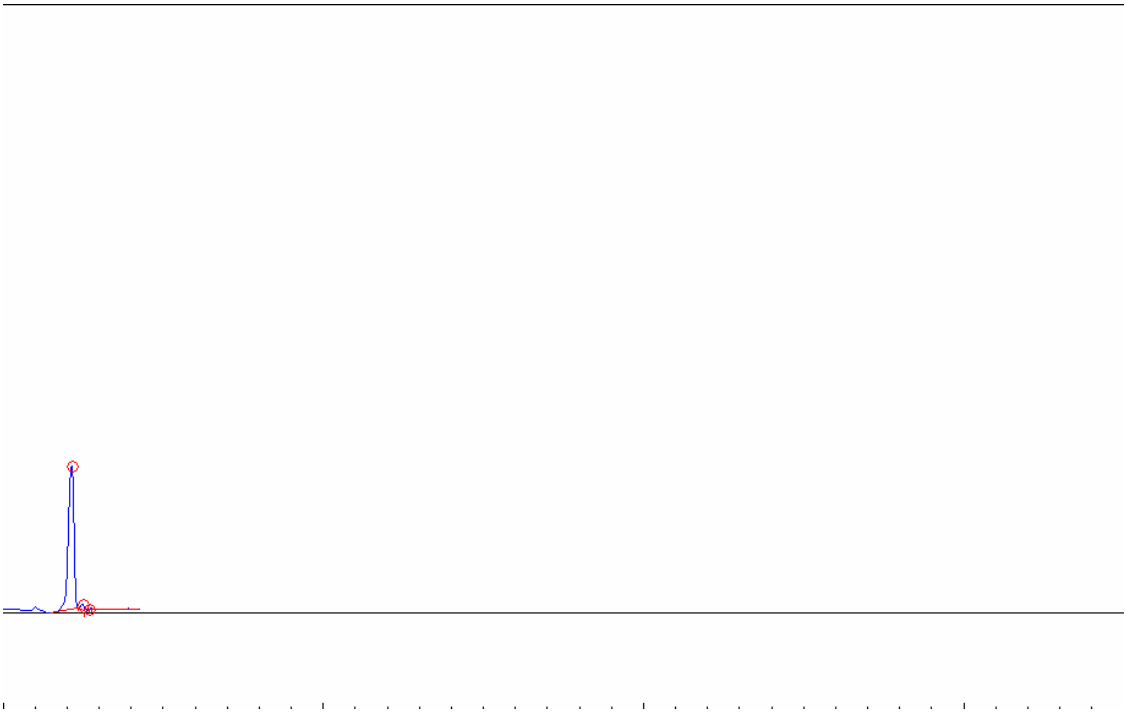


DZ 150 ppm

DZ 200ppm



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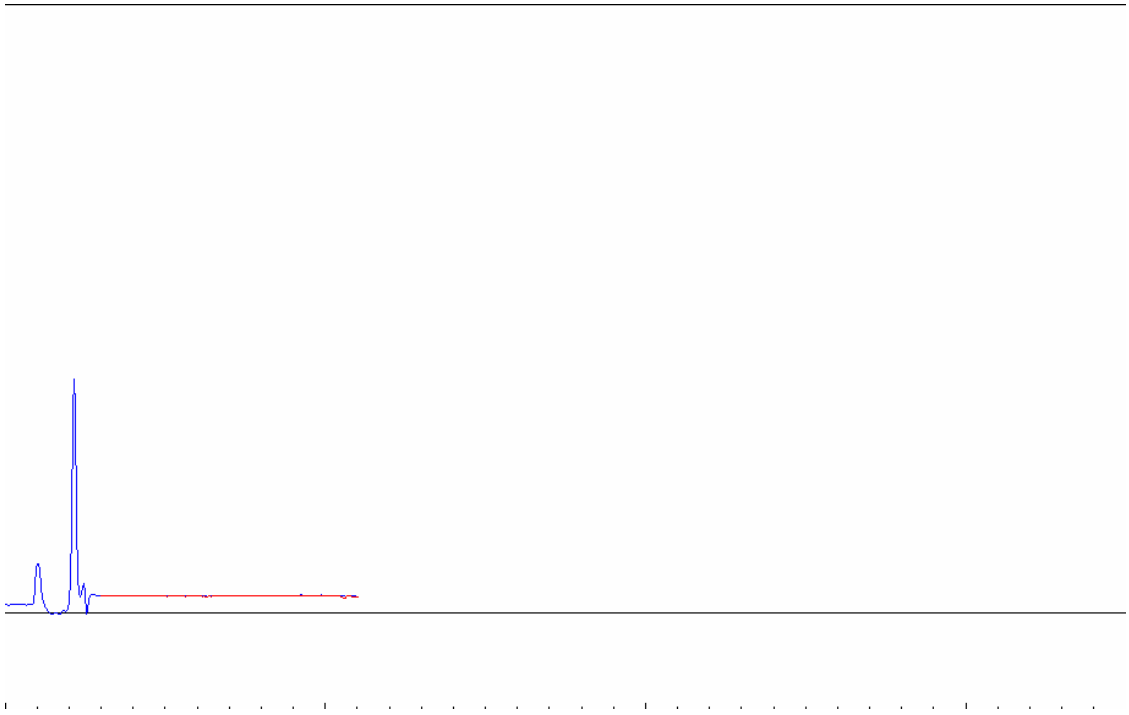

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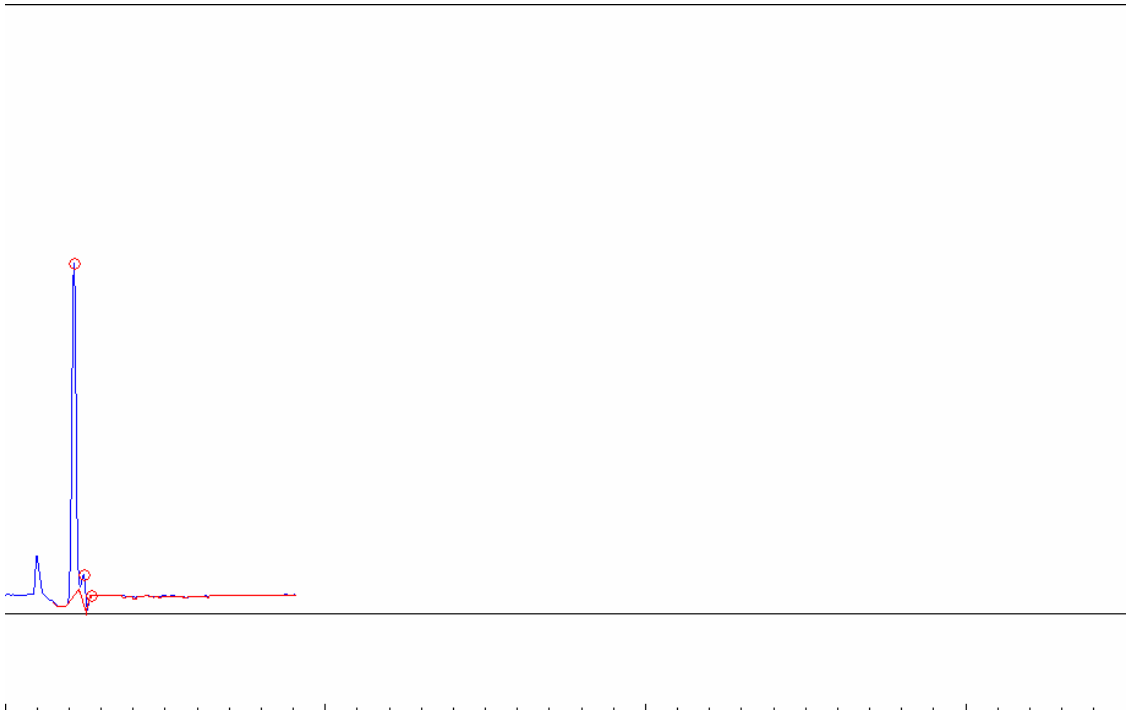
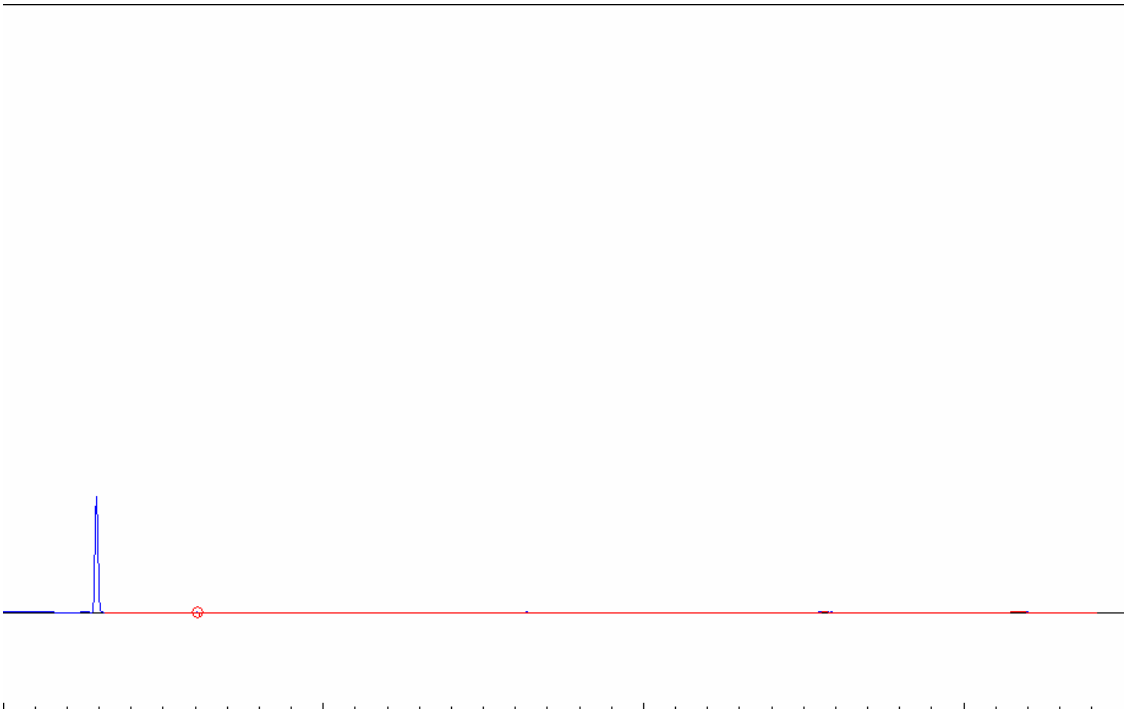


IM 100 ppm

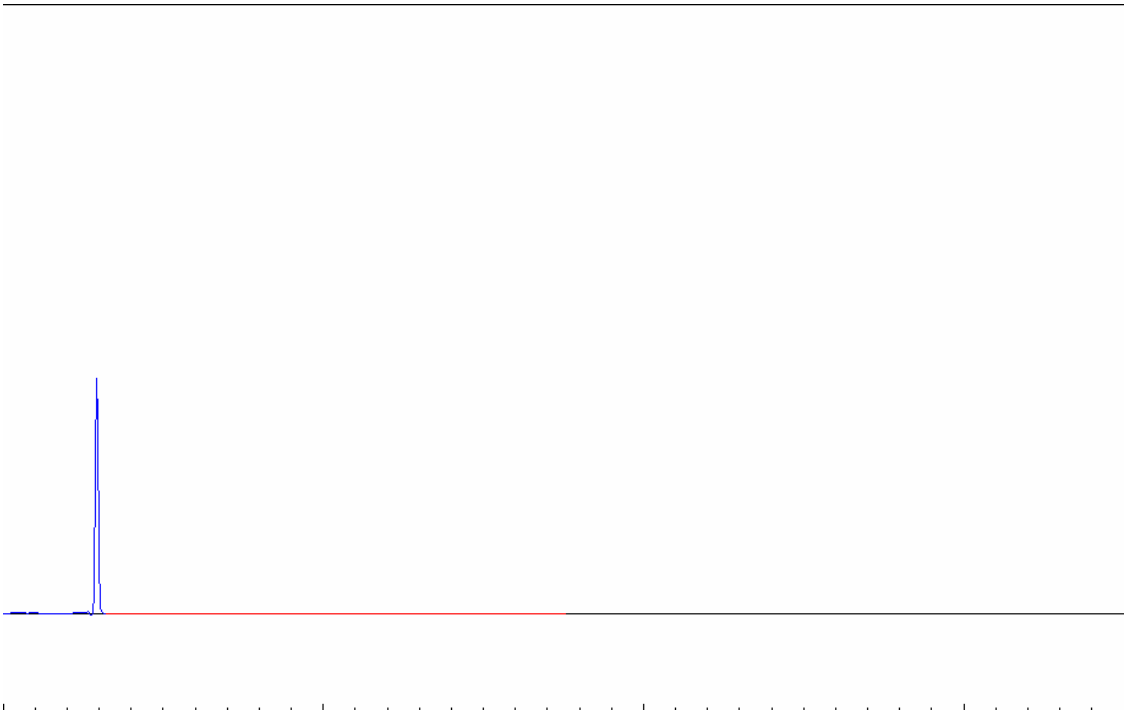
IM 150 ppm

IM 200 ppm

IM 250 ppm



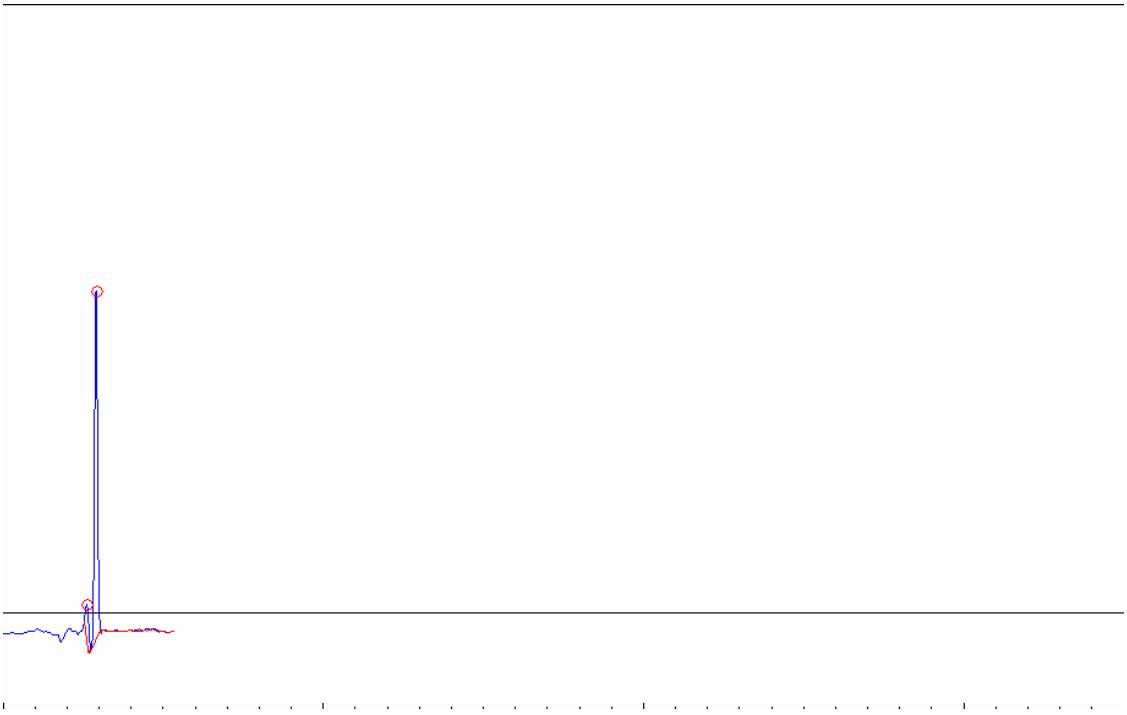
FORM 50 ppm



FORM 100 ppm

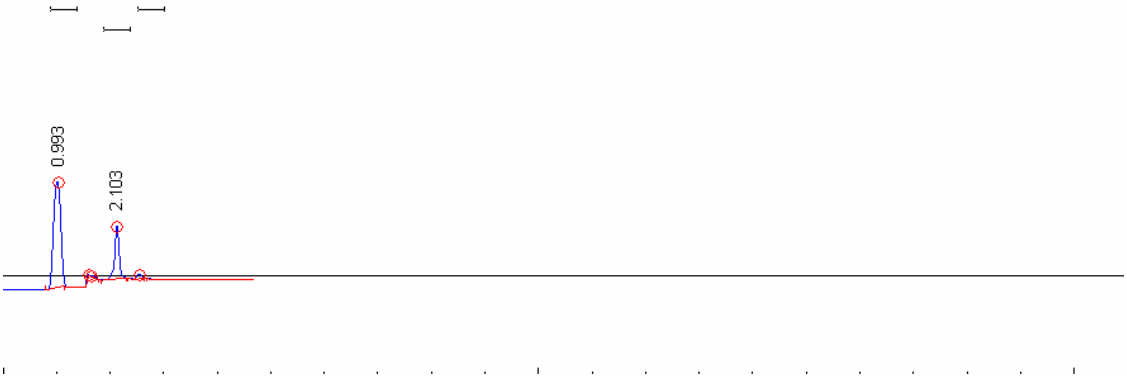
FORM 150 ppm

FORM 200 ppm

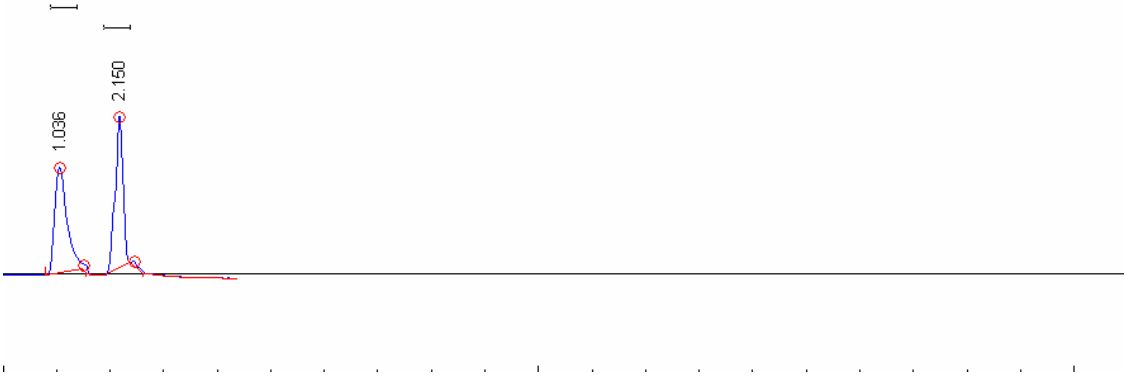


FORM 250 ppm

EXPERIMENTAL SAMPLES



IM D0 Control



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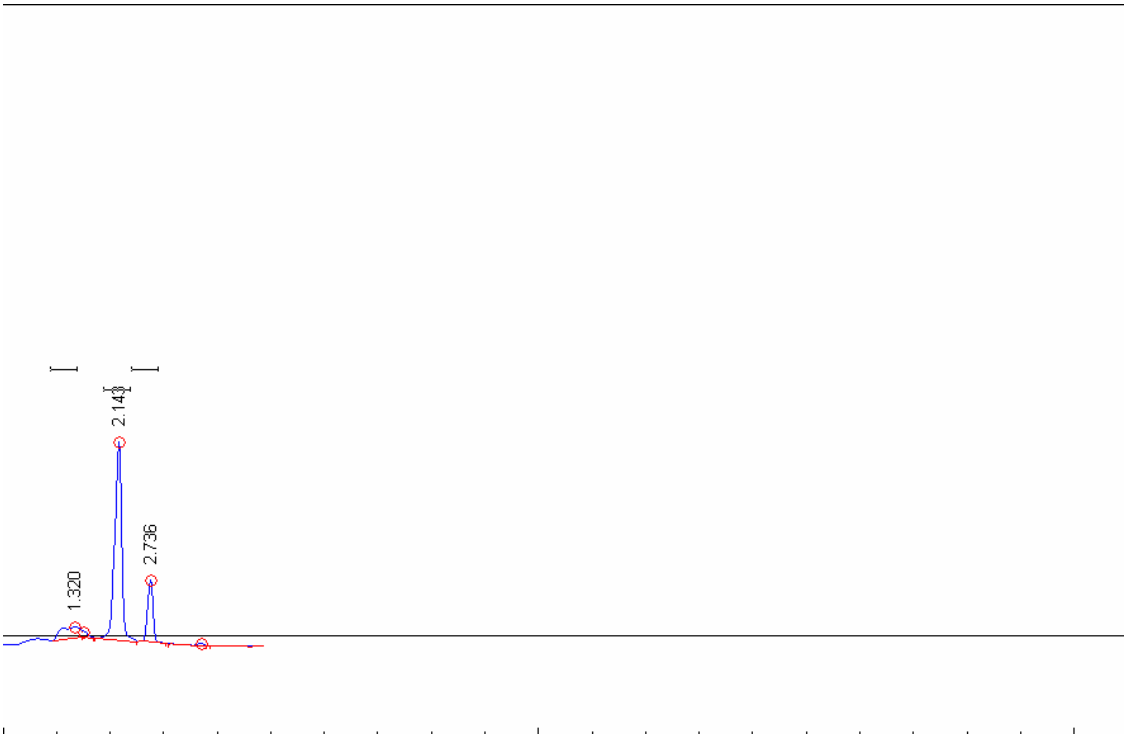
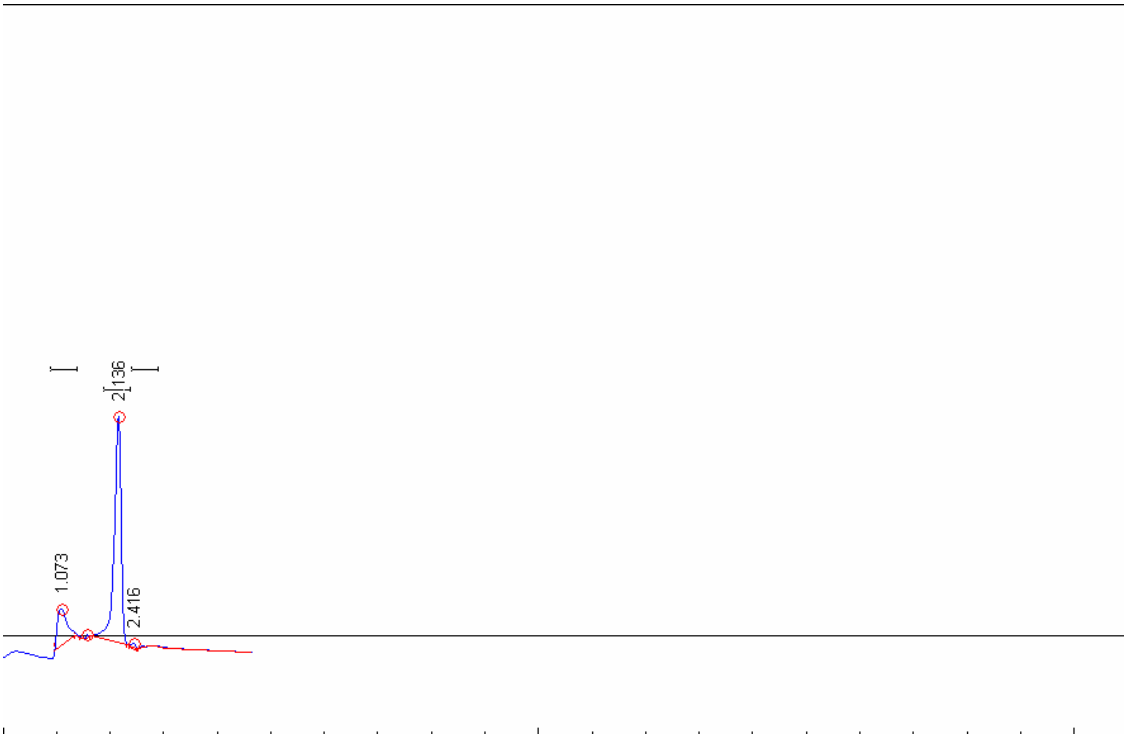
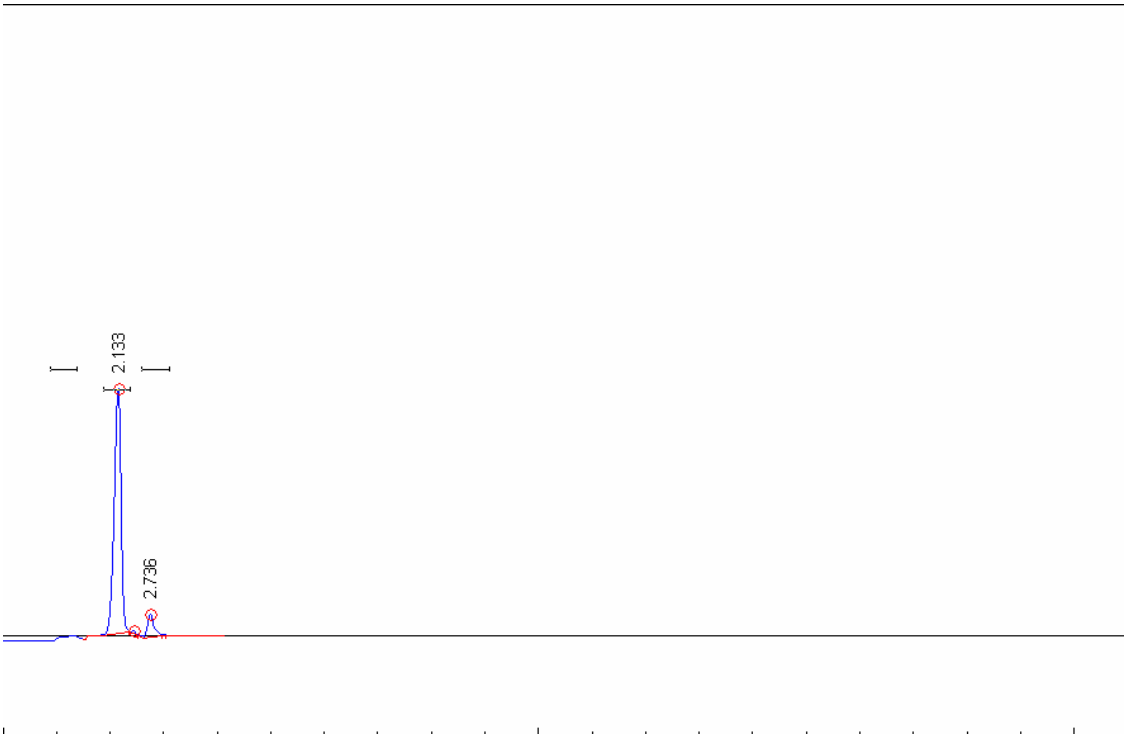


IM D0 (*B. pumilus*)

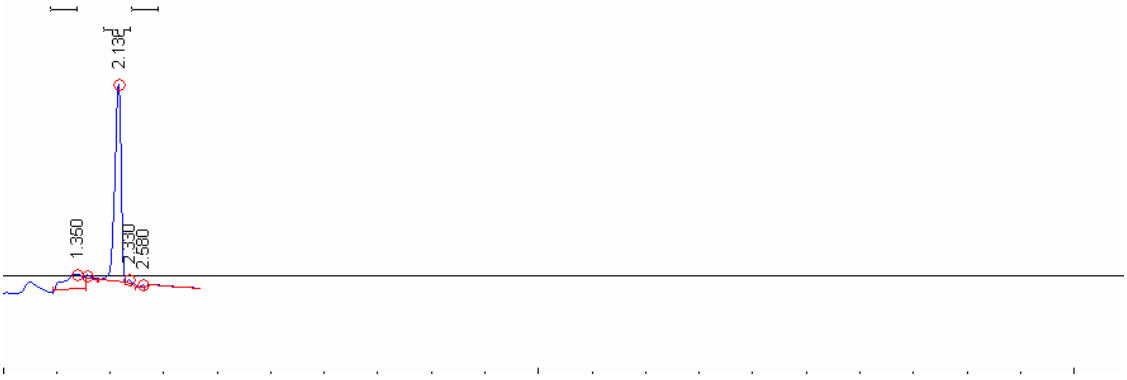


IM D50 (*B. pumilus*)

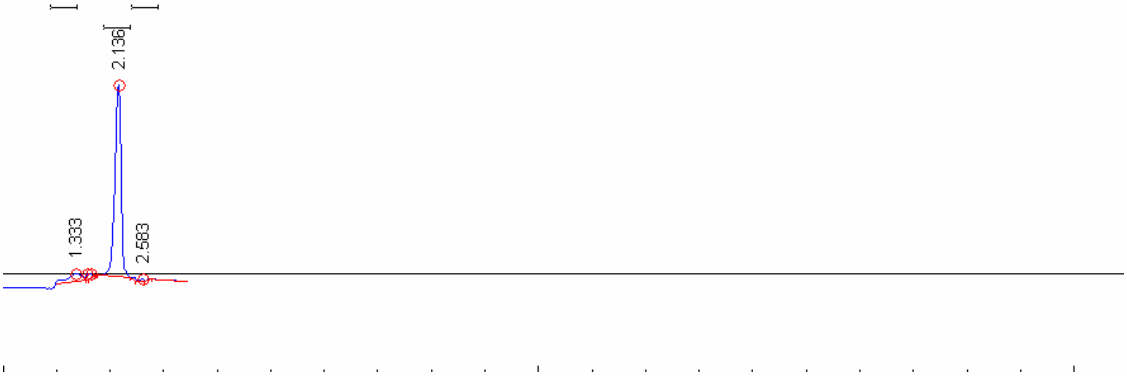


IM D0 (*Citrobacter Freundii*)

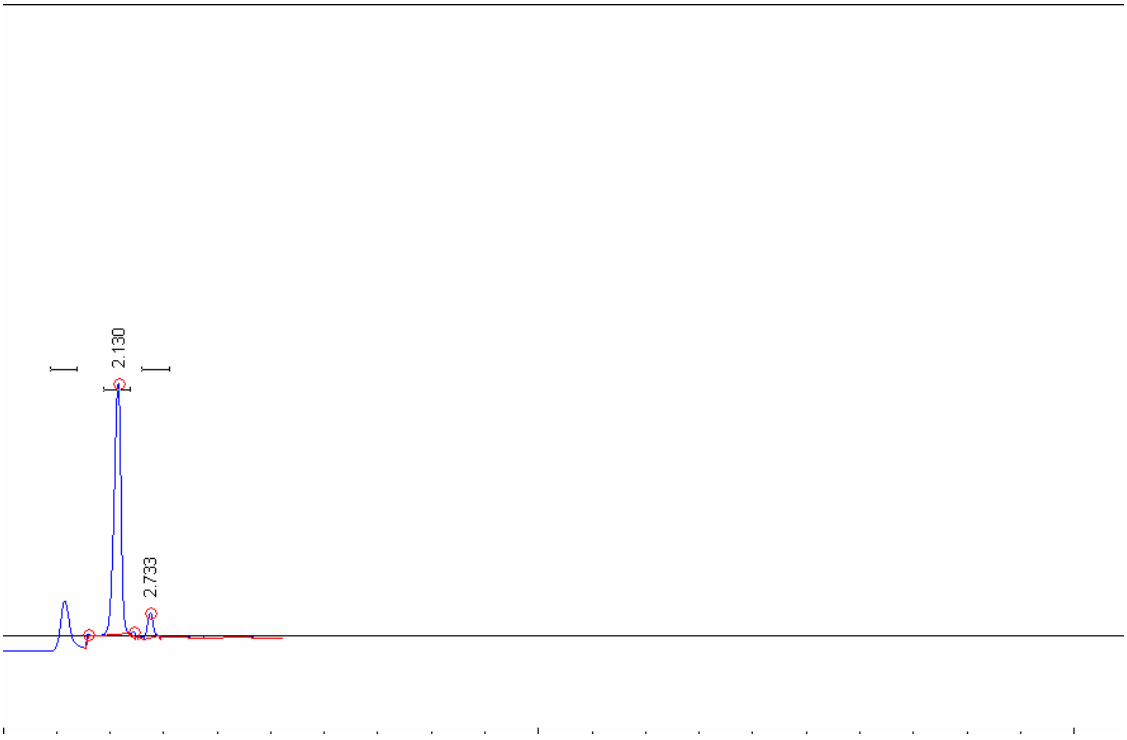
IM D50 (*Citrobacter Freundii*)



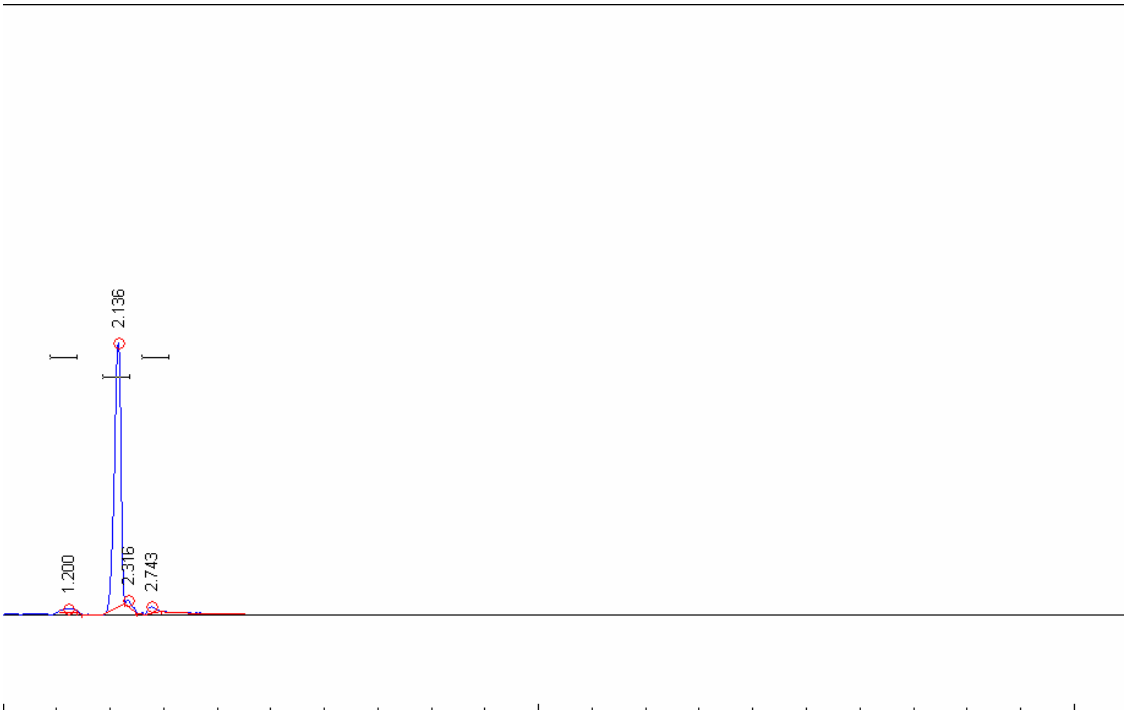
IM D0 Sewage consortium



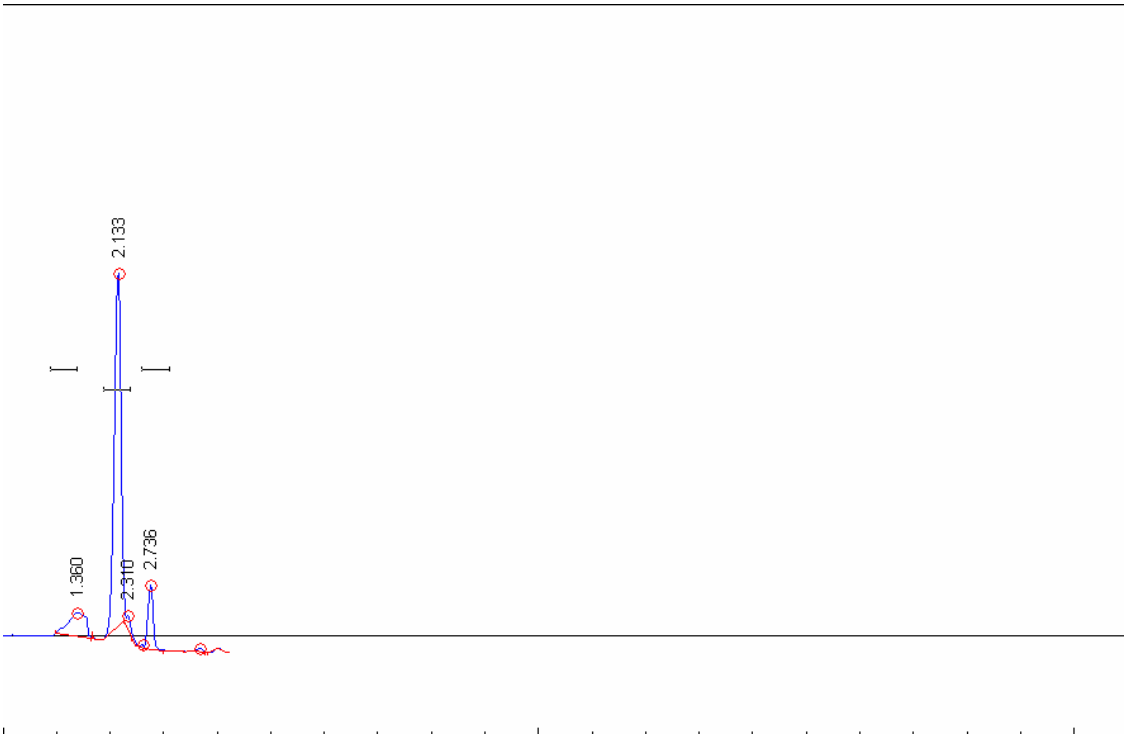
IM D50 Sewage consortium



DZ D0 Control



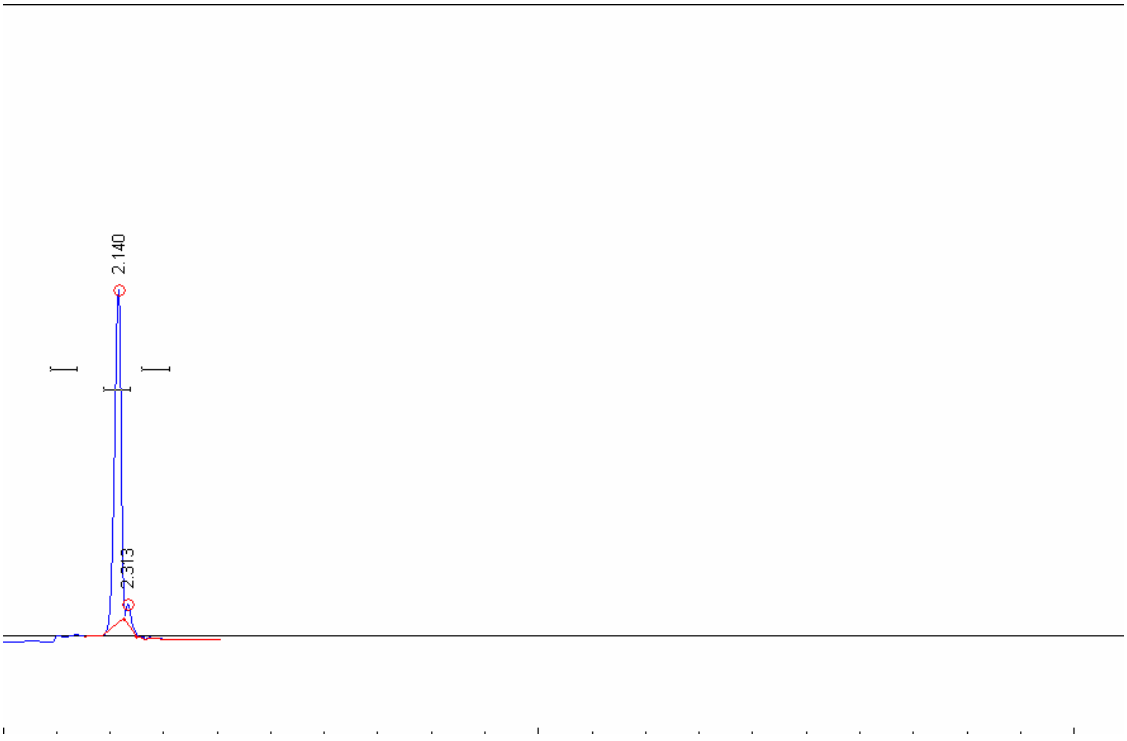
DZ D50 Control



DZ D0 (*B. pumilus*)

DZ D50 (*B. pumilus*)

DZ D0 Sewage consortium



DZ D50 Sewage consortium