PHENOL UTILIZATION AND TOXICITY OF TERNARY MIXTURES OF PHENOL, ZINC AND CADMIUM TO FUNGAL SPECIES ISOLATED FROM HYDROCARBON IMPACTED SOIL

BY

NLEMOLISA, OLUCHI ROSE COLLETTE (B.Sc.)
20144913608

A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL

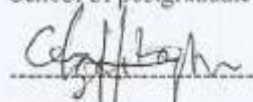
FEDERAL UNIVERSITY OF TECHNOLOGY, OWERRI, NIGERIA.

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE (M.Sc) DEGREE IN ENVIRONMENTAL MICROIOLOGY

NOVEMBER, 2018

CERTIFICATION

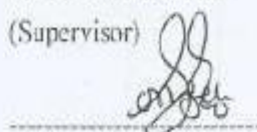
This is to certify that this work "**Phenol utilization and toxicity of ternary mixtures of phenol, zinc and cadmium to fungal species isolated from hydrocarbon impacted soil**" was carried out by **Nlemolisa, Oluchi RoseCollette (20144913608)** in partial fulfillment for the award of Masters of Science degree (M.Sc) in Environmental Microbiology in the Department of Microbiology, School of postgraduate Studies, Federal University of Technology, Owerri, Nigeria.



2/11/2018

Dr. C.E Nwanyanwu
(Supervisor)

Date



2/11/2018

Dr. C.O Akujobi
(Co-Supervisor)

Date.

2/11/2018

Dr. C.O. Nweke
(HOD, Microbiology)

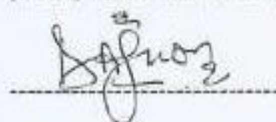
Date

Prof. J.N. Ogbulie
(Dean, School of Biological Science)

Date

Prof. (Mrs) Nnenna. N. Oti
(Dean, Post-Graduate School)

Date



5/11/2018

Prof. D.J.Arotupin
(External Examiner).

Date

DEDICATION

This work is dedicated to Almighty God, and to my father Late Engr. A.C. Nlemolisa who laid the foundation for me, and also to my mother Mrs B.N. Nlemolisa, for her encouragement and support.

ACKNOWLEDGEMENT

Foremost, my gratitude goes to God for His guidance and protection throughout this work.

My sincere gratitude goes to my supervisors, Dr. C.E. Nwanyanwu and Dr. C.O. Akujuobi who gave me their support, and gave suggestions that helped me improve on this work.

I am also grateful to the Head of Department of Microbiology, Dr. C.O. Nweke and the lecturers at the Department for their support and constructive criticism during the presentation of this work.

I would also like to thank Mr C. B. Nzeh, for his assistance during this study and my colleagues and friends for their encouragement.

My sincere gratitude also go to my friend Engr. C.S Ezeh, for his support and encouragement.

My profound gratitude goes to my siblings for their prayers, love, support and encouragement, may God bless you all.

TABLE OF CONTENT

Title i

Certification ii

Dedication iii

Acknowledgement iv

Table of content v

List of Figures x

List of Tables xii

Abstract xv

CHAPTER ONE: INTRODUCTION 1

1.1 Background information 1

1.2 Problem statement 4

1.3 Objectives of study 4

1.4 Justification of study 5

1.5 Scope of study 5

CHAPTER TWO: LITERATURE REVIEW 6

2.1 Soil 6

2.2 Soil and chemical toxicant 6

2.3 Phenol and Toxicity 7

2.4 Biodegradation of phenol 8

2.5 Heavy metals 8

2.5.1 Sources of metals in the environment 9

2.5.2 Heavy metal toxicity 11

2.6 Microbial response used for toxicity assessment 12

2.7 Toxicity 12

2.7.1 Types of toxicity 12

2.7.2 Toxic interaction of chemical mixture 14

2.7.3.1 Types of interaction 14

2.7.4 Toxicity assessment 15

2.8 Dehydrogenase (enzyme) 16

2.9 *Saprochaete* 17

2.10 *Cryptococcus* **17**

CHAPTER THREE: MATERIALS AND METHOD **19**

3.1 Sample collection 19

3.2 Analyses of some physicochemical parameters of the soil sample 19

3.2.1 pH 19

3.2.2 Phosphate content 19

3.2.3 Calcium and Magnesium 20

3.2.4 Organic carbon and organic matter 21

3.2.5 Determination of Total Nitrogen 22

3.2.6 Total Hydrocarbon Content 24

3.2.7 Particle Size Distribution 24

3.2.8 Heavy metal content 27

3.3 Media 27

3.3.1 Nutrient broth 27

3.3.2 Nutrient agar 27

3.3.3 Potato Dextrose Agar (PDA) 28

3.3.4 Mineral Salt Medium (MSM) 28

3.3.5 Mineral salt Broth (MSB) 28

3.3.6 Physiological saline 28

3.4 Reagent 29

3.4.1 2, 3, 5-Triphenyltetrazolium chloride (TTC) 29

3.5 Sample preparation and isolation 29

3.6 Screening of isolates for degradation of TTC 29

3.7 Purification of isolate 30

3.8 Identification of selected isolates 30

3.9 Phenol 30

3.10 Growth on different concentrations of phenol amended mineral salt agar 30

3.11 Hydrocarbon tolerance of the yeasts 30

3.12 Biodegradation of phenol 31

3.12.1Standard calibration curve of phenol 31

3.12.2 Biodegradation assay 33

3.12.2.1 Assay of phenol residue 33

3.12.2.2 Specific growth rate 33

3.12.2.3 Biodegradation rate 34

3.12.2.4 Specific degradation rate 34

3.13 Toxicity assay of phenol and two heavy metals 34

3.13.1 Test chemicals and their preparations 34

3.13.2 Zinc ion (Zn^{2+}) and Cadmium ion (Cd^{2+}) 34

3.13.3 Phenol 34

3.13.4 Preparation of inoculums 35

3.13.5 Enzyme assay in the presence of chemicals 35

3.13.6 Design of experiment for binary and ternary mixtures 42

3.13.7 Estimation of responses 42

3.13.8 Determination of toxicity threshold 42

3.12.9 Analysis of combined effects using toxic index model 43

CHAPTER FOUR: RESULTS AND DISCUSSIONS **45**

4.1 Results 45

4.1.1 Identification of isolates 45

4.1.2 Growth on different concentrations of phenol amended mineral salt agar 45

4.1.3 Hydrocarbon tolerance of the yeast 50

4.1.4 Phenol degradation 53

4.1.5 Toxicity of single chemicals 57

4.1.6 Toxicity of binary mixtures 57

4.1.7 Toxicity of ternary mixtures 80

4.1.8 Equi- effect concentration 80

4.1.9 Toxicity threshold (IC_{50}) 95

4.1.10 Toxic interaction 97

4.2 Discussion 99

CHAPTER FIVE: CONCULSION AND RECOMMENDATIONS **104**

5.1 Conclusion 104

5.2 Recommendations 105

REFERENCE 106

APPENDICES 119

LIST OF FIGURES

Figure	Title	Page
2.1	Sources of heavy metals in the environment	9
4.1	Growth response of the yeast to increase doses of phenol in MSM.	45
4.2	Growth profile and phenol degradation at different initial concentrations by *Crptococcus sp*	48
4.3	Growth profile and phenol degradation at different initial concentrations by *Saprochaete sp*	49
4.4	Toxicity of Zinc as individual chemical on dehydrogenase activity of *Saprochaete sp*	52
4.5	Toxicity of Phenol as individual chemical on dehydrogenase activity of *Saprochaete sp*	53
4.6	Toxicity of Cadmium as individual chemical on dehydrogenase activity *of Saprochaete sp.*	54
4.7	Toxicity of Zinc as individual chemical on dehydrogenase activity of *Cryptococcus sp.*	55
4.8	Toxicity of Phenol as individual chemical on dehydrogenase activity of *Cryptococcus sp.*	56
4.9	Toxicity of Cadmium as individual chemical on dehydrogenase activity of *Cryptococcus sp.*	57
4.10	Toxicity of binary mixture of Zinc and Phenol on dehydrogenase activity of *Saprochaete sp.*	58
4.11	Toxicity of binary mixture of Zinc and Phenol on dehydrogenase activity of *Cryptococcus sp.*	59
4.12	Toxicity of binary mixture of Zinc and Phenol on dehydrogenase activity of *Saprochaete sp.*	60
4.13	Toxicity of binary mixture of Zinc and Phenol on dehydrogenase activity of *Cryptococcus sp.*	61
4.14	Toxicity of binary mixture of Zinc and Phenol on dehydrogenase activity of *Saprochaete sp.*	62
4.15	Toxicity of binary mixture of Zinc and Phenol on dehydrogenase activity of *Cryptococcus*	

sp. 63

4.16 Toxicity of binary mixture of Zinc and Phenol on dehydrogenase activity of *Saprochaete sp.* 64

4.17 Toxicity of binary mixture of Zinc and Phenol on dehydrogenase activity of *Cryptococcus sp.* 65

4.18 Toxicity of binary mixture of Zinc and Cadmium on dehydrogenase activity of *Cryptococcus sp.* 66

4.19 Toxicity of binary mixture of Zinc and Cadmium on dehydrogenase activity of *Saprochaete sp.* 67

4.20 Toxicity of binary mixture of Zinc and Cadmium on dehydrogenase activity of *Saprochaete sp.* 68

4.21 Toxicity of binary mixture of Zinc and Cadmium on dehydrogenase activity of *Cryptococcus sp.* 69

4.22 Toxicity of binary mixture of Zinc and Cadmium on dehydrogenase activity of *Saprochaete sp.* 70

4.23 Toxicity of binary mixture of Zinc and Cadmium on dehydrogenase activity of *Cryptococcus sp* 71

4.24 Toxicity of binary mixture of Zinc and Cadmium on dehydrogenase activity of *Saprochaete sp.* 72

4.25 Toxicity of binary mixture of Zinc and Cadmium on dehydrogenase activity of *Cryptococcus sp.* 73

4.25 Toxicity of ternary mixture of Zinc, Phenol and Cadmium on dehydrogenase activity *Saprochaete sp.* 75

4.26 Toxicity of ternary mixture of Zinc, Phenol and Cadmium on dehydrogenase activity *Cryptococcus sp.* 76

4.28 Toxicity of ternary mixture of Zinc, Phenol and Cadmium on dehydrogenase activity of *Saprochaete sp.* 77

4.29 Toxicity of ternary mixture of Zinc, Phenol and Cadmium on dehydrogenase activity *Cryptococcus sp.* 78

4.30 Toxicity of ternary mixture of Zinc, Phenol and Cadmium on dehydrogenase activity of *Saprochaete sp.* 79

4.31 Toxicity of ternary mixture of Zinc, Phenol and Cadmium on dehydrogenase activity

Cryptococcus sp. 80
4.32 Toxicity of ternary mixture of Zinc, Phenol and Cadmium on dehydrogenase activity of *Saprochaete sp.* 81
4.33 Toxicity of ternary mixture of Zinc, Phenol and Cadmium on dehydrogenase activity *Cryptococcus sp.* 82
4.34 Equieffect (EE_{50}) mixture of Zinc and Phenol on dehydrogenase activity of *Saprochaete sp.* 83
4.35 Equieffect (EE_{50}) mixture of Zinc and Phenol on dehydrogenase activity of *Cryptococcus sp.* 84
4.36 Equieffect (EE_{50}) mixture of Zinc and Cadmium on dehydrogenase activity of *Saprochaete sp.* 85
4.37 Equieffect (EE_{50}) mixture of Zinc and Phenol on dehydrogenase activity of *Cryptococcus sp.* 86
4.38 Equieffect (EE_{50}) mixture of Zinc, Phenol and Cadmium on dehydrogenase activity of *Saprochaete sp.* 87
4.39 Equieffect (EE_{50}) mixture of Zinc and Phenol on dehydrogenase activity of *Cryptococcus sp.* 88

LIST OF TABLES

Table	Title	Page
3.1	Protocol for phenol standard calibration curve	27
3.2	Protocol for obtaining different concentrations used in the assessment of zinc as individual chemical toxicity to phenol-utilizing yeast strains	31
3.3	Protocol for obtaining different concentrations used in the assessment of cadmium as individual chemical toxicity to phenol-utilizing yeast strains	32
3.4	Protocol for obtaining different concentrations used in the assessment of phenol as individual chemical toxicity to phenol-utilizing yeast strains	33
3.5	Protocol for obtaining different concentrations used in the assessment of binary mixtures of zinc and cadmium toxicity to phenol-utilizing yeast strains	34
3.6	Protocol for obtaining different concentrations used in the assessment of binary mixtures of zinc and phenol toxicity to phenol-utilizing yeast strains	35
3.7	Protocol for obtaining different concentrations used in the assessment of ternary mixtures of zinc ,cadmium and phenol toxicity to phenol-utilizing yeast strains	36
4.1	Physicochemical and microbiological analyses of hydrocarbon impacted soil sample	41
4.2	Colonial, morphological and microscopic characteristics of the isolates	42
4.3	Biochemical characteristics of the isolates based on assimilation and fermentation reaction	43
4.4	Hydrocarbon tolerance of the isolates	46
4.4	Biodegradation responses of the isolates.	50

4.5 Toxicity thresholds (IC_{50}) of chemicals as single, binary, ternary and equi-effects mixtures on dehydrogenase activity of the test yeasts. 90

4.6 Toxic Interactions of the binary, ternary and equi- effect mixtures of the test chemicals on Dehydrogenase activity of the yeast 92

ABSTRACT

The present study was carried out to determine phenol utilization and toxicity of ternary mixtures of zinc, cadmium and phenol to fungal species isolated from hydrocarbon impacted soil. The yeasts isolated from the hydrocarbon impacted soil were identified as *Saprochaete sp.* and *Cryptococcus sp.* The biodegradation phenol potential of *Saprochaete sp.* and *Cryptococcus sp.* was studied using mineral salt broth supplemented with phenol with concentration ranging from 0-1000 mg/l. Investigation shows that the yeasts were able to utilize almost all the introduced quantity (1000 mg/l) of phenol as sole source of carbon and energy within a period of 3 to14 days. The toxicity was determined based on the inhibition of dehydrogenase activity of *Saprochaete sp.* and *Cryptococcus sp*. The toxicity of chemicals and their mixtures was evaluated in the concentration range of 0-16 mM for phenol, 0-5 mM for zinc and 0-0.4 mM for cadmium. The binary and ternary mixture ratios were evaluated. The toxicity thresholds (IC50) were estimated using 3-parameter logistic dose-response model. The IC50 shows that cadmium has the highest toxic effect on the yeasts with IC50 value of 0.075 mM for *Saprochaete sp.* and 0.09 mM for *Cryptococcus sp.* The binary and ternary toxicity of the mixtures on the enzyme activity of the yeasts was evaluated with toxic index (TI) model. Modulation of the toxic interactions by the components of the mixtures through synergistic, additive and antagonistic interactions on the heavy metals and phenol were possible against the dehydrogenase activity of the phenol-utilizing yeasts. However the toxic effects of phenol and heavy metals would depend on their relative amount present in the environment.

Keywords: Biodegradation, dehydrogenase activities, phenol, heavy metals, toxic index model.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Soil is normally considered as the fine earth which covers land surfaces as a result of the in situ weathering of rock materials or the accumulation of mineral matter transported by water, wind, or ice. The distinctive feature of soil is that to this weathered mineral material is added organ sz ic material (Nortcliff *et al.,* 2012). This organic material may be both living and dead. The dead organic matter will include little altered and freshly added dead plant roots and leaf and other plant litter, dead fauna, and organic material in various stages of decomposition from little modified relatively fresh materials to the complex decomposed material called humus. It is this mixture of mineral and organic material which gives the soils their distinctive characteristics (Nortcliff *et al.,* 2012). Across the surface of the earth there are many different types of soil which reflect, at least in part, varying combinations of mineral and organic matter and their differing responses–both separately and often in complex association–to different environmental conditions. Indeed soil (and the soil constituents), together with the plant life it supports, the rock on which it lies, and the climate it experiences, forms a finely balanced system (Nortcliff *et al.,* 2012).

The advent of modern technology and industrialization, environmental pollution of the natural resources by chemicals has become a serious concern. Most of these pollutants are released into the environment through industrial, agricultural, and pharmaceutical waste as a mixture of organic and inorganic pollutants and they cause destructive effects on living systems such as mutation, cancer, poisoning, and so on and so forth. The industrial effluents usually consist of a number of contaminants in varying concentrations which are generally harmful to the environment and living things (El nahash *et al*., 2009). Organic pollutants released to the environment as a result of these activities include phenol, polycyclic aromatic hydrocarbons, and heavy metals. Among those pollutants, phenolic compounds pose a threat to the environment because of its toxicity and stability of its bioaccumulation (Teixeira *et al*., 2015). Phenol and phenolic compounds are

widely used in production of dyes, plastics, resins, composition of oils and so on and so forth (Rigo and Alegre, 2004). They are considered as one of the priority pollutants by the Environmental Protection Agencies (Wang *et al.*, 2012; Cravotta and Brady, 2015). These have been reported as highly toxic and hazardous to living organisms (ATSDR, 1998).

These phenolic compounds are often found in waste water from coal-gasification, coke-oven batteries, refinery and petrochemical plants and other industries, such as synthetic chemicals, herbicides, pesticides, antioxidants, pulp and paper, photo developing chemicals, and so on and so forth (Kavita and Palanivelu, 2004; Jayachandran and Kunhi, 2008). Phenols released into the environment from different industries are of high concern because of their potential toxicity. Many of the phenolic compounds are carcinogenic and are listed in the U.S. Environmental Protection Agency's priority pollutant (Zhang and Wiegel, 1990; Neumaan *et al*., 2004) and these toxic and xenobiotic chemicals cause challenging problems for the environment due to their recalcitrant in nature.

Phenol is slightly solvable in water due to its ability to make hydrogen bonds with water (Morrison and Boyd, 1992). Today, there is a lot of anxiety regarding the existence of toxic chemical substances like nitrate, selenium, mercury, cadmium and phenol in water, which could enter the human body through consumption of aquatic animal. Freshwaters are usually contaminated with sewages from factories. Most of the rivers have been turbid and obscured due to entry of sewages, chemicals, oily materials and other extraneous material. Nowadays, rivers, gulf, lake and oceans are the most contaminated waters resource, respectively. Phenol and its compounds are one of most important pollutant of the environment (especially, water). Due to the toxic properties, including permeabilisation of cellular membranes and cytoplasm coagulation, phenolic contaminants can damage sensitive cells and cause profound health and environmental problems (Tziotzios *et al.,* 2005).

As a result of human activities like metalliferous mining and smelting, agriculture, waste disposal or industry discharge, a variety of metals such as silver, arsenic, gold, cadmium, cobalt, chromium, copper, mercury, nickel, lead, selenium and zinc, which can produce harmful effects on microorganisms are released in the

environment. Some metals are required by plants in very small amounts for their growth and optimum performance. However, the increasing concentration of several metals in soil and waters due to industrial revolution has created an alarming situation for human life and aquatic biota (Girma, 2015).

Heavy metals are a dangerous group of soil pollutants that cannot be naturally degraded and as such accumulate in different parts of the food chain (Šmejkalová *et al.,* 2003). Although some are essential for growth, others can be harmful to living organisms by forming complexes with protein molecules thereby rendering them inactive (Kim, 1985; Azza *et al*., 2009). Heavy metals have been shown to have detrimental effects on microorganisms even at low concentrations (Kim, 1985) by affecting microbial growth, morphology, biochemical activities and enzymatic processes (Nweke *et al.,* 2007). Heavy metals have been reported to be powerful inhibitors of biodegradation activities (El. Deeb and Altalhi, 2009). Heavy metals having relatively high density are toxic at low concentration (Iram *et al.,* 2013). Microorganisms and plants are usually used for the removal of heavy metals. Processes involving the use of microorganisms to reduce pollutant concentration in an environmental media are known as bioremediation which is a natural process and it is increasingly considered for clean-up of metal contamination and polluted ecosystem (Doelman *et al.,* 1994).

Metal toxicity cause serious morbidity and mortality (Surajana and Manas, 2009). Furthermore, Jin *et al.* (2011) reported that the bioavailability can be improved by addition of organic nutrients (such as manure, compost, biosolids) to the soil, which condition the soil and increases the fertility of soil. In order to make the environment healthier for human beings, contaminated water bodies and land need to be remediated or clean up to make them free from heavy metals and trace elements. Use of microorganisms and plants for remediation purposes is thus, a possible solution for heavy metal pollution since it uses sustainable remediation technologies to clean up and re-establish the natural condition of soil (Wasiullah*, et al.,* 2015).

However, introduction of heavy metals into the soil causes considerable modification of the microbial community, despite their vital importance for the growth of microorganisms at relatively low concentrations (Doelman *et al*., 1994). The modification of the microbial make up is mainly brought about by exerting an

inhibitory action through blockage of essential functional groups, displacement of essential metal ions or modification of active conformations of biological molecules. The response of microbial communities to heavy metals depends on the concentration and availability of heavy metals, it is a complex process which is controlled by multiple factors, such as type of metal, the nature of the medium and microbial species.

1.2 Problem statement

Phenols and heavy metals are widely distributed as environmental pollutants due to its common presence in industrial, agricultural and pharmaceutical waste. These toxic and xenobiotic chemicals cause challenging problems for the environment due to their recalcitrant nature. Many of these chemicals have been shown to have detrimental effects on microorganisms even at low concentrations (Kim, 1985) by affecting microbial growth, morphology, biochemical activities and enzymatic processes (Nweke *et al.,* 2007). In view of the magnitude of public health challenges associated with pollution, coupled with the pressing need for alternative, sustainable, cheaper and more environmental-friendly method of cleaning-up of phenol and heavy metals, which is bioremediation. Therefore, the purpose of this study is to investigate the phenol utilization of phenol by fungi speices, and to ascertain the toxicity of phenol and heavy metals (phenol, zinc and cadmium) as single, binary and ternary mixtures on fungi species isolated from hydrocarbon impacted soil.

1.3 Objective of study

The main objective of this study is to investigate phenol utilization, hydrocarbon tolerance and toxicity of ternary mixtures of phenol, zinc and cadmium to fungi species isolated from hydrocarbon impacted soil.

The specific objectives are:

(a) To isolate and identify fungal species from hydrocarbon impacted soil,

(b) To evaluate the ability of the fungal isolates to degrade phenol.

(c) To access the hydrocarbon tolerance of the fungal species.

(d) To ascertain the toxicity of phenol, zinc and cadmium as individuals, binary and ternary mixtures on dehydrogenase (enzyme) activity of fungal species.

(e) To evaluate the toxic interactions of the chemical mixtures on dehydrogenase (enzyme) activity of fungal species.

1.4 Justification of study

On daily basis, large volumes of pollutants are released into the environment through industrial, agricultural and pharmaceutical waste as a mixture of organic and inorganic pollutants. Some organisms are able to tolerate these metals at varying concentrations. Although conventional techniques applied for the treatment of these phenols and heavy metals are mechanical and physic-chemical properties, often associated with biological treatment and may be formed by separation through gravity, centrifugation, application of coagulants, flotation, filtration, adsorption with activated carbon and others (Santo *et al.,* 2012), Ionic exchange, excavation and solidification/ stabilization, these technologies are suitable to control contamination but, not permanently remove heavy metals (Bahi *et al.,* 2012). One way to reduce treatment cost, while re-establishing the use of microorganism for bioremediation of the environment. Fungi have advantages over bacteria such as fungal hyphae that can penetrate to reach pollutants (Husaini *et al.,* 2008).

It is imperative to embark on this study to ascertain the suitability of fungal species in phenol utilization as well as the toxicity of zinc, cadmium and phenol on fungal species.

1.5 Scope of study

The research work entails collection of hydrocarbon impacted soil from Orji mechanic village, Isolation and characterization of fungi species isolated from the hydrocarbon impacted soil, degradation of phenol by the fungi species, hydrocarbon tolerance of the fungal species, toxicity of phenol, zinc and cadmium as individual, binary and ternary mixtures on dehydrogenase (enzyme) activities of fungal species and the toxic interaction of the chemical mixtures on dehydrogenase (enzyme) activity on fungal species.

CHAPTER TWO

LITERATURE REVIEW

2.1 Soil

Soil is a mixture of minerals, organic matter, gases, liquids, and countless organisms that together support life on Earth. Soil is a natural body called the pedosphere which has four important functions: it is a medium for plant growth; it is a means of water storage, supply and purification; it is a modifier of Earth's atmosphere; it is a habitat for organisms; all of which, in turn, modify the soil. Soil is called the Skin of the Earth (Austin, 1953) and interfaces with the lithosphere, the hydrosphere, the atmosphere, and the biosphere (Chesworth, 2008). Soil consists of a solid phase of minerals (the soil matrix) and organic matter, as well as a porous phase that holds gases (the soil atmosphere) and water (the soil solution) (Voroney and Heck, 2015). Soil is a product of the influence of climate, relief (elevation, orientation, and slope of terrain), organisms, and its parent materials (original minerals) interacting over time. Soil continually undergoes development by way of numerous physical, chemical and biological processes, which include weathering with associated erosion. Given its complexity and strong internal connectedness soil has been considered as an ecosystem by soil ecologists (Ponge, 2015).

Soil is the most abundant ecosystem on Earth, but the vast majority of organisms in soil are microbes, a great many of which have not been described (Amber, 2008). There may be a population limit of around one billion cells per gram of soil, but estimates of the number of species vary widely from 50,000 per gram to over a million per gram of soil (Roesch *et al.*, 2007). The total number of organisms and species can vary widely according to soil type, location, and depth (Roesch *et al.*, 2007).

2.2 Soil and chemical toxicants

Phenols, sometimes called phenolics, are a class of chemical compounds consisting of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group. The simplest of the class is phenol, which is also called carbolic acid (C_6H_5OH). Phenolic compounds are classified as simple phenols or polyphenols based on the number of phenol units in the molecule (Amorati and Valgimigli, 2012). Phenolic compounds are

synthesized industrially; they are also produced by plants and microorganisms, with variation between and within species (Hättenschwiler and Vitousek, 2000). Although similar to alcohols, phenols have unique properties and are not classified as alcohols (since the hydroxyl group is not bonded to a saturated carbon atom). They have higher acidities due to the aromatic ring's tight coupling with the oxygen and a relatively loose bond between the oxygen and hydrogen. Some phenols are germicidal and are used in formulating disinfectants. Others possess estrogenic or endocrine disrupting activity (Hättenschwiler and Vitousek, 2000).

2.3 Phenol toxicity

Phenol is a monosubstituted aromatic compound and is recognized as a major environmental pollutant. It is produce through natural and anthropogenic processes. It is found in nature as a constituent of coal tar and is also formed during natural decomposition of organic material (WHO, 1994; EPA, 2002). However, the major part of phenol present in the environment is due to industrial use. Currently, the largest use of phenol is as an intermediate in the production of phenolic resins, which are used in the plywood, adhesive, construction, automotive and appliance industries (EPA, 2002). Phenol may persist in air, sea water or surface water, soil or sewage. Now, the associated problem due to phenol is that when it is present in waste water, even in low concentrations, it can be toxic to some aquatic species (Rittmann and McCarty, 2001).

Phenol poses several toxic cellular effects (EPA, 2002). Membrane toxicity is the most pronounced effect (Sikkema *et al*., 1995). Phenol toxic effect on membranes is mainly due to its lipophilicity. Thus, phenol tends to accumulate into lipophilic layer of the cell membranes disturbing its integrity. Hence, it is not surprising to use phenol in disinfectants (EPA, 2002).

Biodegradation of phenol is often inhibited by toxicity exerted at high concentration (Okpokwasili and Nweke, 2006). Phenol is a membrane-damaging microbiocide causing changes in the lipid-to-lipid and lipid-to protein ratios in the membrane, membrane permeability and activity of membrane-associated proteins (Heipieper *et al*., 1992). The loss of cytoplasmic membrane integrity results in disruption of energy transduction, disturbance of membrane barrier function, inhibition of membrane protein function and

subsequent cell death. Due to its toxicity to microorganisms, phenol may often cause the breakdown of wastewater treatment systems by inhibition of microbial growth (Ren and Frymier, 2003).

2.4 Biodegradation of phenol

Inspite of phenolic toxic properties, a number of microorganisms can utilize phenol under aerobic conditions as sources of carbon and energy (Chen *et al*., 2006). Biodegradation technologies most often take advantage of the ability of various bacteria to clean the environment, bioremediation is constantly expanding. Fungi are famous for their wide incidence and the outstanding capacity of degrading complex and inert natural products like lignin, chitin and cellulose (Supriya and Neehar, 2014). Fungi adopt more easily than bacteria and are capable to grow in extreme conditions, like nutrient deficiency, low pH, limited water supply, and so on and so forth (Atagana and World, 2000). And not on the least, there comes the ability of fungi to survive in the presence of various xenobiotics that turn to be toxic to a number of other microorganisms. Metabolism of aromatic compounds, such as phenol and its derivatives, has been extensively studied in prokaryotic microorganisms (Watanabe *et al*., 1998). Particularly much information has been collected about bacterial species of *Pseudomonas* genus (Seker *et al*., 1997). Number of individual representatives of the genera *Candida, Rodotorula* and *Trichosporon,* are capable of metabolizing aromatic compounds (Mac Gillivray and Shiaris, 1993). The specific enzymes responsible for biodegradation occupy an important place in these investigations. In 15 strains belonging to *Fusarium, Aspergillus, Penicillium* and *Graphium* genera the presence of phenol hydroxylase and catechol 1, 2-dioxygenase activity in cells cultivated on a phenol-containing medium has been confirmed. These findings demonstrate that catechol oxidation follows the ortho – pathway of breaking the aromatic ring (Supriya and Neehar, 2014).

2.5 Heavy metals

Metals in general are class of chemical element that forms lustrous solid that are good conductors of heat and electricity. Heavy metals are defined in a number of ways based on cationic hydroxide formation, hard soft acid and bases, and more recently, association with eutrophication and environmental toxicity (Wasiullah, *et al*., 2015).

Heavy metals also known as trace elements had been reported to come from weathering of rock (natural) minerals which may be increased substantially by anthropogenic activities such as industrial and agricultural activities and enter our environment (Kabata-Pendias, 2000). The soil serves as a long term 'save' sink for heavy metals such as lead, cadmium, zinc, copper and nickel. Heavy metals such as copper and zinc in the soil are essential trace elements for plants and animals but excessive concentrations through external additions can damage the overall soil fertility and agricultural productivity. These metals are toxic at soil concentration above normal level (Inuwa *et al*., 2007). Chromium is taught to be essential metal are required for enzyme catalysis, molecules transport, protein structure, charged neutralization and control of osmotic pressure. An important point to note is that although these metals are essential for microbial growth and metabolism, at high concentrations, essential metal can become toxic. The toxic metals include those with no biological functions and these include silver (Ag), cadmium (Cd), slicion (Sn), lead (Pb), aluminium (Al), mercury (Hg) and metalloids. Toxic metals exert their properties via displacement of essential metals from their normal binding sites on biological molecules (Bruins *et al.,* 2000).

2.5.1 Sources of metals in the environment

Heavy metals occur naturally in the environment from pedogenetic processes of weathering of parent materials and also through anthropogenic sources (Figure 2.1). The most significant natural sources are weathering of minerals, erosion and volcanic activity, while the anthropogenic sources depend upon human activities such as mining, smelting, electroplating, use of pesticides and phosphate fertilizer discharge, as well biosolids (for example, livestock manures, composts, and municipal sewage sludge), atmospheric deposition, (Wuana and Okieimen, 2011). The heavy metals essentially become contaminants in the soil and water environment because of their excess generation by natural and man-made activities, transfer from mines to other locations where higher exposure to humans occurs, discharge of high concentration of metal waste through industries, and greater bioavailability (Wasiullah, *et al*., 2015).

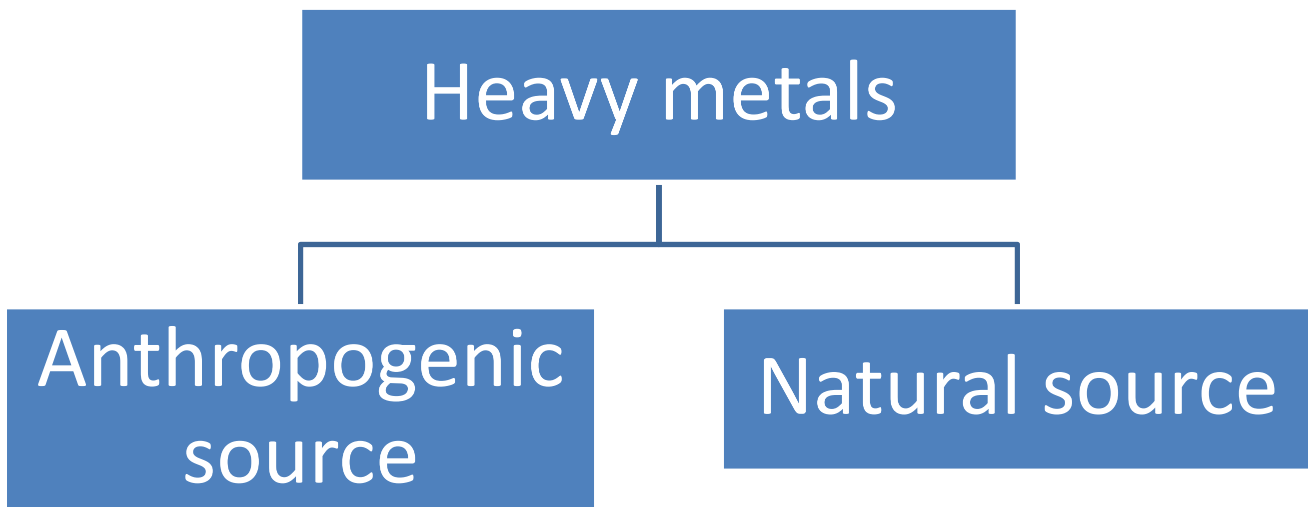


Figure 2.1. Sources of heavy metals in the environment (Wasiullah, *et al*., 2015)**.**

2.5.2 Heavy metal toxicity

Heavy metals pollution is increasing and poses a serious threat to environment and organisms. The levels of metals in all environments, (air, water and soil) are increasing in some cases to toxic levels, with contributions from wide variety of industrial and domestic sources. Metal contaminated environments pose serious threat to health and ecosystems. The most abundant pollutants in waste water and in sewage are heavy metals (Hong *et al.*, 2002).The environmental pollution by heavy metals comes from anthropogenic sources such as smelters, mining, power stations and the application of pesticides containing metal, fertilizer and sewage sludge and the irresponsible disposal of wastes by various industries (Meghraj and Daneshwar, 2013).

Heavy metal toxicity to microorganisms has been assessed by a number of studies. In general, it has been shown that the presence of heavy metals can inhibit or cease microbial growth (Sani *et al*., 2003), while it often results in prolonged lag times of microbial growth (Gikas, 2007). However, low concentrations of selected heavy metals may act as growth stimulants. This is because some heavy metals, at low concentrations, are utilized by microorganisms in various biochemical pathways as enzymes cofactors, usually in the form of metalloproteins (Nies 1999). The effects of heavy metals on microorganisms depend on the type, speciation, and concentration of the heavy metal and type of microorganisms. Additionally, a number of environmental parameters, such as pH, buffers, and presence of other metallic and nonmetallic ions or organic substances (Lee and Lustigman 1996), have also been shown to affect the above interaction. Many of the heavy metals are toxic even at very low concentrations; arsenic, cadmium, chromium, copper, lead, mercury, nickel, selenium, silver, zinc and so on and so forth. are not only cytotoxic but also carcinogenic and mutagenic in nature (Salem, *et al.,* 2000).

Both essential and nonessential heavy metals, at relatively high concentrations, may interact with the microorganisms at three levels: They may be coupled on the microbial membrane, causing irreversible damage such as loss of membrane integrity, absorbed in the cytoplasm oxidizing vital enzymes or inactivating microbial organelles, or affect the genetic material of the microbial cell by reacting directly with

DNA . Typically, heavy metal concentrations above trace levels result in prolonged lag time and/or reduced growth rate, and the duration of the lag is observed to be dependent on the metal concentration (Sani *et al*., 2003).

2.6 Microbial response used for toxicity assessment

Several microbial responses used in assessing toxicity of chemical include; Production of light by photo-bacteria or bioluminescence bacteria, cell multiplication, production of metabolic products such as acids, vitamin, CO_2, protein, and so on and so forth, cell mobility or mobilization of cell, substrate consumption.

The estimation of respiratory activities has been used primarily to assess toxicity of chemicals to bacteria (Okolo *et al*., 2007). The oxygen uptake rates and reduction of redox indicators are followed polarographically and spectophotometrically respectively.

2.7 Toxicity

Toxicity is the degree to which a substance can damage an organism. Toxicity can refer to the effect on a whole organism, such as an animal, bacterium, or plant, as well as the effect on a substructure of the organism, such as a cell (cytotoxicity) or an organ such as the liver (hepatotoxicity) (Merriam-Webster Dictionary, 1828). By extension, the word may be metaphorically used to describe toxic effects on larger and more complex groups, such as the family unit or society at large. Sometimes the word is more or less synonymous with poisoning in everyday usage.

A central concept of toxicology is that the effects of a toxin are dose-dependent; even water can lead to water intoxication when taken in too high a dose, whereas for even a very toxic substance such as snake venom there is a dose below which there is no detectable toxic effect (Reuters, 2015). Toxicity is species-specific, making cross-species analysis problematic. Newer paradigms and metrics are evolving to bypass animal testing, while maintaining the concept of toxicity endpoints (AltTox.org, 2012).

2.7.1 Types of toxicity

There are generally four types of toxic entities; chemical, biological, physical and radiation:

Chemical: Chemical toxicants include inorganic substances such as, lead, mercury, hydrofluoric acid, and chlorine gas, and organic compounds such as methyl alcohol, most medications, and poisons from living things (Matsumura and Ananthaswamy, 2004). While some weakly radioactive substances, such as uranium, are also chemical toxicants, more strongly radioactive materials like radium are not, their harmful effects (radiation poisoning) being caused by the ionizing radiation produced by the substance rather than chemical interactions with the substance itself (Matsumura and Ananthaswamy, 2004).

Biological: Disease-causing microorganisms and parasites are toxic in a broad sense, but are generally called pathogens rather than toxicants (Matsumura and Ananthaswamy, 2004). The biological toxicity of pathogens can be difficult to measure because the "threshold dose" may be a single organism. Theoretically one virus, bacterium or worm can reproduce to cause a serious infection. However, in a host with an intact immune system the inherent toxicity of the organism is balanced by the host's ability to fight back; the effective toxicity is then a combination of both parts of the relationship (Matsumura and Ananthaswamy, 2004). In some cases, for example, cholera, the disease is chiefly caused by a nonliving substance secreted by the organism, rather than the organism itself (Matsumura and Ananthaswamy, 2004). Such nonliving biological toxicants are generally called toxins if produced by a microorganism, plant, or fungus, and venoms if produced by an animal.

Physical: Physical toxicants are substances that, due to their physical nature, interfere with biological processes. Examples include coal dust, asbestos fibers or finely divided silicon dioxide, all of which can ultimately be fatal if inhaled (Matsumura and Ananthaswamy, 2004). Corrosive chemicals possess physical toxicity because they destroy tissues, but they're not directly poisonous unless they interfere directly with biological activity. Water can act as a physical toxicant if taken in extremely high doses because the concentration of vital ions decreases dramatically if there's too much water in the body (Matsumura and Ananthaswamy, 2004). Asphyxiant gases can be considered physical

toxicants because they act by displacing oxygen in the environment but they are inert, not chemically toxic gases (Matsumura and Ananthaswamy, 2004).

2.7.2 Toxic interaction of chemical mixtures

Chemicals may interact with one another and modify the magnitude and sometimes the nature of the toxic effect. Various interactions can take place when organisms are exposed to mixtures of pollutants and may be described as additive, synergistic, or anatagonistic (Preston *et al*., 2000). Co-occurring toxicants may interact to produce unexpected physiological effects (Smadar and Mordechai, 2009). Interactions may take place in the toxicokinetic phase (that is processes of uptake, distribution, metabolism and excretion) or in the toxicodynamic phase (that is effects of chemicals on the receptor, cellular target or organ) (Groten *et al*., 2001).

2.7.3.1 Types of interactions

Additive effects – This is the sum of the effects of the chemicals involved in the reaction. This arise when the toxicity of the mixture is equal to the sum of the toxicities of the individual component (that is 1+1=2) (Preston *et al*., 2000). This usually occurs with chemicals that are similar in structure, so they work well as a team - kind of like a superhero and a sidekick. An example of an additive interaction would be taking both aspirin and acetaminophen, which is the active ingredient in drugs like tylenol (Preston *et al*., 2000).

Synergistic effects – These are when the sum of the effects is more than each chemical individually. This can create dangerous situations because each chemical is designed to work well on its own. For example, alcohol and acetaminophen are a dangerous combination in the body. This is because both are processed in the liver, and each puts a lot of strain on the liver (Preston *et al*., 2000). Synergistic effect occur when the toxic effect of the mixture is greater than expected (that is 1+1>2).

Antagonistic effects- These are when the net effect of the chemical reaction is zero. Antagonistic effects are important because this is where we get antidotes for poisons. Anti-venom for snakebites is an example of an antagonistic effect (Preston *et al*., 2000) so is the combination of caffeine and alcohol (Preston *et al*., 2000). This is when the effect of the mixture is less than expected (1+1<2).

Potentiating effects- this is when one chemical enhances the effect of another chemical. Some chemicals are not toxic on their own, but when they are in the presence of some other chemicals, they become toxic (Preston *et al*., 2000).

Hormesis effect- Hormesis is a biphasic dose-response relationship, regarded as an adaptive function. The hormetic response is characterized by a mild stimulation (30-60%) of the considered function, at low concentrations, and by the inhibition of the same function at higher dose (Preston *et al*., 2000). Many phylogenetically distinct organisms and biological systems exposed to a wide range of stimuli show a dose-dependent biphasic response (Calabrese, 2008).In an unpredictable variable environment, the hormetic process allows a single individual to overcome a stress condition of low/medium intensity.

2.7.4 Toxicity assessment

Toxicity can be measured by its effects on the target (organism, organ, tissue or cell). As a matter of fact, because individuals typically have different levels of response to the same dose of a toxic substance, a population-level measure of toxicity is often used which relates the probabilities of an outcome for a given individual in a population (Kapanen and Itavaara, 2001). Toxicity assessment establishes the relationship between the contaminants of concern and the receptor. Commonly used measures of toxicity include; Lethal concentration (LC_{50}) which is the concentration of a toxicant that kills 50% of a test population for a given exposure duration. Effective concentration (EC_{50}) which is the concentration of a given compound that reduces the specified effect to half that of the original response, inhibitory concentration (lC_{50}) is analogous

to (EC_{50}). Minimum inhibitory concentration (MIC) is the lowest concentration of the toxicant needed to produce an inhibitory effect. LC_{50} and EC_{50} are measures of acute toxicity (Kapanen and Itavaara, 2001).

2.8 Dehydrogenase (enzyme)

Dehydrogenases are a subclass of the class of enzymes labeled "oxidoreductases." Oxidoreductases, in general, catalyze oxidation and reduction reactions. Any enzyme that transfers an electron from one molecule to another is considered an oxidoreductase (Donald, 2006). Most oxidoreductases enzymes are dehydrogenases, although reductases are also common. Oxidation-reduction reactions are essential to growth and survival of organisms, as the oxidation of carbons produces energy (BioWiki, 2016). Energy-producing reactions can drive forward the synthesis of important energy molecules, such as ATP in glycolysis. For this reason, dehydrogenases have pivotal roles in metabolism. Dehydrogenases (enzymes) transfer electrons from the substrate to an electron carrier; what carrier is used depends on the reaction taking place. Redox indicators are used as artificial electron acceptor in dehydrogenase assay to determine intracellular flux of electrons from electron donors to acceptors in the presence of toxicants. The most widely used redox indicator is 2, 3, 5-triphenyltetrazolium chloride (TTC) which is reduced to red coloured 2, 3, 5-triphenyl formazan (TPF) by microbial dehydrogenase (Nweke and Okpokwasili, 2010).

The general reaction is: $TTC \xrightarrow{+2e^- \; +2H^+} TPF$

Dehydrogenase activity measurement is based on quantification of TPF produced from the reduction of TTC. The intensity of the red colouration of the formazan is the function of the viability of the cells. Reduction of TTC has been used as a measure of microbial growth or viability in the presence or absence of toxicant (Gabrielson *et al*., 2002).

2.9 *Saprochaete* sp

Saprochaete is ubiquitous yeast found in environmental sources such as soil, water, air, plants and dairy products (Pamidimukkala *et al*., 2017). It is also a part of the normal microbial flora of the human skin, gastrointestinal tract and respiratory tract. It is non-fermentative, non-encapsulated, urease-negative ascomycetous yeast which is thermotolerant and can grow in the presence of cycloheximide (Mazzocato *et al.,* 2014; Arendrup *et al.,* 2014). The yeast grew as white coloured colonies on Sabouraud's dextrose agar. Microscopic examination of the lactophenol cotton blue mount from the colony showed anneloconidia, typical of *Saprochaete capitata* along with arthroconidia, blastocondia, true hyphae and pseudohyphae, distinguishing the yeast from the closely related *Trichosporon* and *Geotrichum* species (Umabala *et al.,* 2017).

2.10 *Cryptococcus* sp

Cryptococcus (Greek for "hidden sphere") is a genus of fungi, which grow in culture as yeasts. The sexual forms or teleomorphs of *Cryptococcus* species are filamentous fungi in the genus *Filobasidiella*. The name *Cryptococcus* is used when referring to the yeast states of the fungi (Ross and Taylor, 1981). The cells of these species are covered in a thin layer of glycoprotein capsular material that has a gelatin-like consistency, and that among other functions, serves to help extract nutrients from the soil (Ross and Taylor, 1981). However, the *Cryptococcus neoformans* capsule is different, in being richer in glucuronic acid and mannose, having O-acetyl groups (Ross and Taylor, 1981) and functioning as the major virulence factor in cryptococcal infection and disease (Casadevall and Perfect, 1998). *Cryptococcus* , a heterothallic basidiomycete, aerobic, Gram positive, encapsulated yeast. It is oval to round in shape, that reproduce by multilaterial budding. These yeast are non- fermentative and are characterize by their ability to assimilate inositol as a sole carbon source, to produce urease (Marianna and Anna Maria, 2009).

Cryptococcus neoformans, the etiological agent of cryptococcosis, has been associated with soil (Bunting *et al.,* 1979) and pigeon droppings (Emmons, 1955). The organism was first isolated from soil by Emmons in 1951 (Emmons, 1951). Four years later, he reported the isolation of this yeast from a majority of specimens (pigeon nests and pigeon droppings) collected from several premises. Emmons concluded that the pigeon was not a host for *Cryptococcus neoformans* and described the yeast-pigeon droppings relationship as an "adventitious saprophytic occurrence in a suitable medium" (Emmons, 1955).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection

Hydrocarbon contaminated soil sample was collected from Orji mechanic Village. The soil sample was collected using a sterile metal cylindrical tool (soil auger) at a depth of 5 cm below the soil surface. The sample was transferred into a sterile polyethylene bag and immediately delivered to the laboratory for analysis.

3.2 Determination of physicochemical parameters of the soil sample

3.2.1 pH

Soil pH was determined using pH meter (Metler Delta 340 pH meter model). The soil sample's suspension in distilled water, with soil-water ratio of 1: 2.5 was prepared according to the modified method of Mc Lean (1982). 5 g of sieved, air dried soil sample was weighed into a clean dried 20 ml test tube. To this, 12.5 ml distilled water was added. The mixture was then stirred vigorously for 15 seconds and allowed to stand for 30 minutes. The instrument was then standardized using freshly prepared buffer at pH 4.0 (phthalate buffer) and pH 7.0 (phosphate buffer) by dipping the electrode into each of the buffer solution. The electrode was then rinsed with deionized water, cleaned with an absorbant cotton wool and dipped into the sample. The pH of the sample was then read off on the display panel.

3.2.2 Phosphate content

Available phosphorus was determined using the modified Bray No.1 method (Olsen and Sommers, 1982). Five gram (5 g) of air dried soil was weighed into a 125 ml plastic extraction bottle. To this 100 ml of 0.5 N $NaHCO_3$ extraction solution pH 8.5 was added. The extraction vessels were placed on an oscillating mechanical shaker for 30 minutes at 22^oC. Then the suspension was filtered immediately within 1 minute through a Whatman 42 filter paper (it was re-filtered if filtrate was cloudy). To a 5 ml aliquot of filtrate, 5 ml of 0.5 N H_2SO_4 was added de-gas by shaking under vaccum, and 11 ml of deionized water was added. Four

milliliters (4 ml) of a freshly prepared Murphy-Riley ascorbic acid solution was added and mixed. The concentration of phosphorous was then determined in a spectrophotometer (VIS spectrophotometer 721D, Life Assistance Scientific INST. CO) adjusted and operated in accordance with manufacturer's instructions. Absorbance was read at a wavelength of 815 nm after 30 minutes of adding the Murphy-Riley ascorbic acid solution. Standards were treated in the way as the sample and the readings used to draw a standard curve from where the test was read.

Calculations

Soil bicarbonate available phosphorous is reported to the nearest 0.1 mg/kg

Soil PO_4 P mg/kg = (PO_4 P mg/l in extract- blank) x 20

3.2.3 Measurement of calcium and magnesium

The calcium and magnesium contents (combined) of the sample were determined using the method by El-Mahi *et al.* (1987). Two grams (2 g) of the sample were introduced into a conical flask, 30ml of ammonium acetate were added to it and the flask shaken for five minutes and decanted. Thirty milliliters (30ml) of 0.5N hydrochloric acid were added and the mixture agitated for five minutes in an upright position. The solution was filtered by passage through a Whatman No.1 fitter paper and the filtrate was collected. Twenty milliliters (20ml) of the filtrate was pipetted out into a 150 ml conical flask and 50 ml of distilled water were added. Ten milliliters (10ml) of buffer solution, 10 drops each of $NH_2OH.HCL$, potassium ferrocyanide, triethanolamine (TEA) and eriochrome black T indicator were added and the mixture was titrated with a standard ethylene diamine tetra acetic acid (EDTA) to a permanent blue colour.

Measurement of calcium content alone

The method used by El-Mahi *et al. (*1987) was adopted. Five milliliters (5ml) of the filtrate were pipetted out. Ten drops each of $NH_2OH.HCL$, potassium ferrocyanide and triethanolamine and three drops of 10% sodium hydroxide solution were added to raise the pH to 12. Five drops of calcon indicator were added and the mixture was titrated against EDTA till the colour changed from red to blue and the value taken.

Measurement of magnesium content alone

This was measured using the method of El-Mahi *et al.* (1987). The magnesium content was determined from the calculation:

Ca + Mg together – Ca alone = Mg

3.2.4 Organic carbon and organic matter

Organic carbon was measured using modified Walkley and Black method (Gaudette *et al.*, 1974), which was based on the exothermic heating and oxidation of organic matter with potassium dichromate and concentrated sulphuric acid, followed by back-titration with ferrous ammonium sulphate using phenyl-amine as an indicator.

The air dried soil samples were first ground and sieved through a 0.42 mm mesh sieve. Then soil sample 1 g was weighed into a dry 500 ml conical flask. Then to the 500 ml flask, 10 ml, 1 N $K_2Cr_2O_7$ was accurately added and the flask swirled gently to disperse the soil in the solution. Immediately, 20 ml concentrated H_2SO_4 was added to the flask. The flask was then swirled until the soil and the reagent are mixed. A 200°C thermometer was then inserted and the mixture heated, while swirling the flask and the contents over a gas burner and gauze until the temperature reached 135°C. The flask was then set aside to cool slowly on an asbestos sheet in a fume cupboard. Two blanks (without soil) were prepared in the same way to standardise the FeSO4 solution. When cool (20-30 minutes), the mixture was diluted to 200 ml with deionised water and then titrated with $FeSO_4$ using the "ferroin" indicator. Ferroin was dissolved 1.485 g O-phenanthroline monohydrate and 0.695 g ferrous sulphate in approximately 80 ml deionised water, then diluted to 100 ml. Three drops of ferroin indicator was added to the flask and then titrated with 0.4 N $FeSO_4$. As the end point was approached, the solution took on a greenish colour and then changes to a dark green. At this point, the ferrous sulphate was added drop-by-drop until the colour changed sharply from blue-green to reddish-grey.

Calculations

1 ml of 1 N dichromate solution is equivalent to 3 mg of carbon.

Where the quality and normality of the acid/ dichromate mixture used are as stated in the method, the percentage carbon is determined from the following:

Organic carbon (%) = (0.003 g x N x 10ml x (1- T/s) x 100)/ODW

= 3 (1- T/S)/W

Where:

N = Normality of K_2CrO_7 solution

T = Volume of $FeSO_4$ used sample titration (ml)

S = Volume of $FeSO_4$ used in blank titration (ml)

ODW = Oven-dry sample weight (g)

Determination of organic matter

The percentage organic matter was calculated from the percentage organic carbon by the equation, percentage (%) organic matter = organic carbon (%) x 1.724

3.2.5 Determination of total nitrogen

Total nitrogen was determined using the macro kjeldahl method of Walkley and Black (1934). Nitrogen was oxidized and converted to ammonium with an acid and the quantity of ammonium produced was then determined by distillation with an alkali. The process was carried out in two stages namely; digestion of soil with H_2SO_4 to convert nitrogen to ammonium and determination of quantity of ammonium.

Ten grams of air dried soil was weighed into a dry 500 ml Macro-kjeldahl flask. To the flask, 20 ml of water was added and swirled for 5 mins. The flask was then allowed to stand for 30 mins. Then, 10 g K_2SO_4, 1 g $CuSO_4.5H_20$, 0.1 g Se and 30 ml conc. H_2SO_4 were added and mixed by swirling. The flask was then heated cautiously in a fume chamber with intermittent swirling until the digest turned light green or gray in colour. Heating was continued from this stage for another one hour. The flask was then allowed to cool and then slowly, while shaking the flask, 100 ml of tap water was added, and the solution transferred to a clean flask for distillation.

Into a 500 ml flask on which the 150 ml level has been marked, 50 ml of 4% boric acid (H_3BO_3) solution and 3 drops of mixed indicators were added. The flask was then placed under the condenser of the distillation apparatus so that the end of the condenser was dipped into the solution inside. A small piece of litmus paper was dropped into the flask containing the diluted digest. Then 125 ml of 45% NaOH was added to the flask carefully by pouring down the side of the flask so that the alkali reached the bottom of the flask without mixing appreciably with the digest. The flask was then attached to the condenser and swirled to mix the contents. The litmus paper now turned blue, indicating that the solution was alkaline. The solution was then distilled, while regulating the heat to minimise bumping, until about 150 ml of the distillate was collected. The distillate was then titrated with 0.05N H_2SO_4 until the colour changed from green through greyish-blue to pink. A blank was also prepared and titrated as the experinmental.

Calculation :

Milliequivalents of N in the sample = ml 0.05N H_2SO_4 used – blank value x normality of H_2SO_4

Total % N = ml 0.05N H_2SO_4 x normality x F

F (correction factor) = 0.14

3.2.6 Total hydrocarbon content (THC)

Total hydrocarbon content (THC) was obtained through spectrophotometric method by Tanee and Albert. (2015). One gram (1 g) of soil sample was oven –dried at 70°C for 24 h. The THC content of the dried soil sample was extracted using chloroform as extraction solvent and its concentration measured using spectrophotometer.

3.2.7 Particle Size Distribution

Particle size distribution was determined by the method as described by Stewart *et al.* (1974). The dispersant used was calgon (mainly sodium hexametaphosphate) prepared at 5 % (w/v) by dissolving 50 g of calgon in 100 ml distilled water and adding $NaCO_3$ to bring the final pH to 9.0 and diluting to 1 liter. Fifty grams (50 g) of air-dired 2 mm sieved sample was dired and placed into polyethene bag. Twenty five milliters (25 ml) of 5% calgon and 400 ml of water were added to the sample and thorough dispersed by shaking for 2 h on an oven shaker. This was then transferred to a liter of measuring cylinder and diluted to mark. This was further stirred with a glass stirring rod. A bouyoucus hydrometer was introduced (20 s before reading) and the reading was taking after:

$$\text{Clay (\%)} = \frac{A(gl^{-1}) \times 100}{(50 - \text{Moisture wt})\,g} - 1$$

$$\text{Silt + Clay (\%)} = \frac{B\,(gl^{-1}) \times 100}{(50 - \text{Moisture wt})\,g} - 1$$

Where 1 = Calgon correction

$$\text{Silt (\%)} = \text{Silt + Clay (\%)} - \text{Clay (\%)}$$

Where A = hydrometer reading (gl^{-1}) after 5 h

B = hydrometer reading (gl^{-1}) after 4 min 48 sec. (silt + clay)

If (50 – moisture weight)g soil are dispersed in 1 liter

The texture class was also determined using the ‘textured triangular diagram’ (Loganathan, 1984). Results of particle size analysis provide percentages for the three size classes. The results are also used to assign a particular textural class to each sample using the textural triangular diagram (Fig. 3.1).

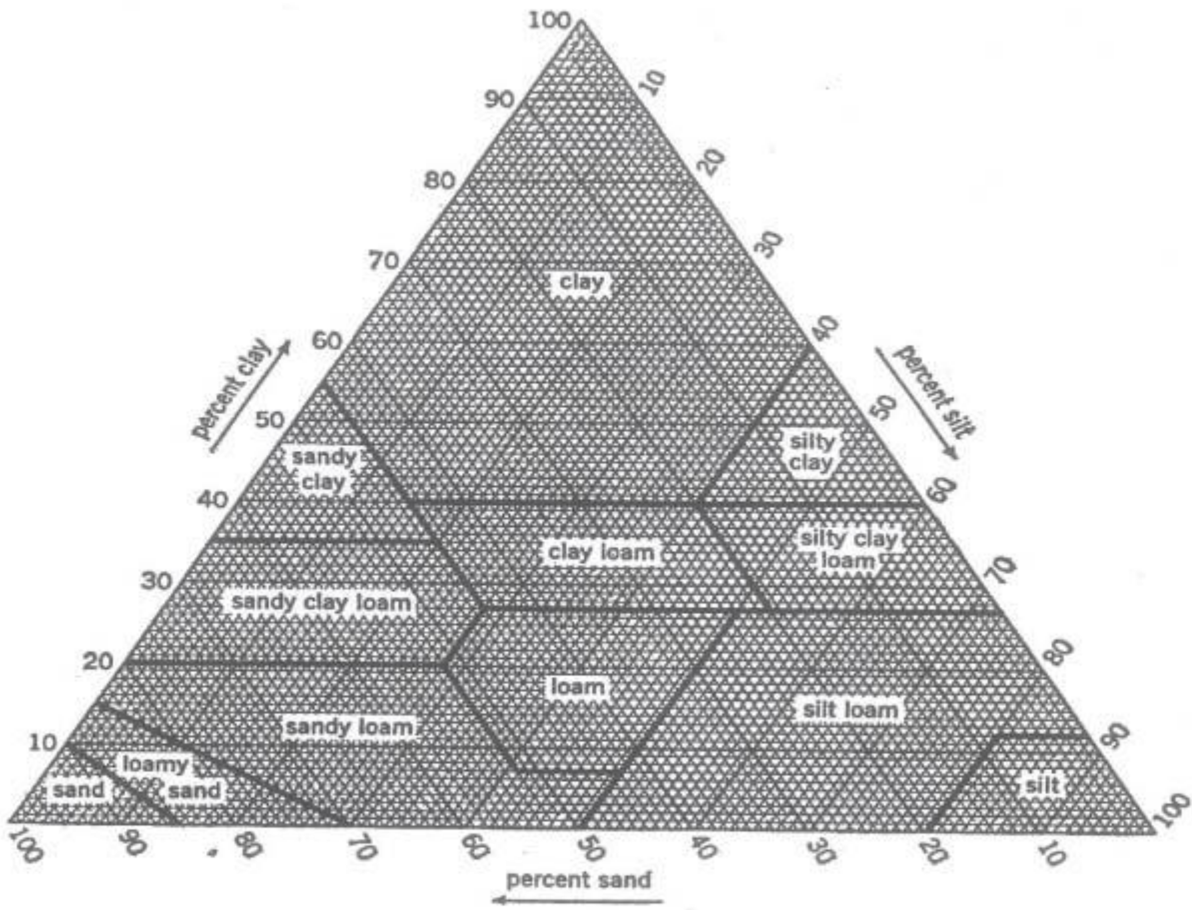


Fig. 3.1: Textural triangular diagram (Uzukwu *et al.,* 2011)

3.2.8 Heavy metal content

Zinc and copper

The digestion method as described by Francek *et al*., (1994) was adopted for the extraction of trace metals. One gram each of air-dried soil sample was crushed to fine powder in an agate mortar and digested in 10 ml of 1:1 concentrated HNO_3. The mixture was evaporated to near dryness on a hot plate and then cooled. This procedure was repeated with a 15 ml solution of 1:1 concentrated HCl. The extracts were filtered with Number forty (40) Whatman filter paper and then made up to 100 ml volume with 2% HNO_3. Solutions of the sample and blanks were run using Atomic Absorption Spectrometer (AAS).

Cadmium and lead

One gram of each of the sieved soil samples was digested using the nitric/perchloric acid digestion procedure, as described by Odu *et al.* (1986). The concentrations of heavy metals, Pb and Cd were determined using atomic absorption spectrophoto-meter (UnicamSolaar32 model) following the standard procedures as given in APHA (1995).

3.3 Media

3.3.1 Nutrient broth

This was prepared according to the manufacturer's procedure; thirteen grams (13 g) of the nutrient broth powder was dissolved in 1000 ml of sterile water. The medium was sterilized by autoclaving at 121℃ for 15 mins at 15 pounds per square inch (psi) and the medium was allowed to cool. The medium was used in toxicity assay which allowed for microbial growth and for the growth of the isolates.

3.3.2 Nutrient agar

This was prepared according to the manufacturer's procedure; 5.6 g of nutrient agar was weighed and dissolve in 200 ml of water. It was sterilized by autoclaving at 121℃ for 15 mins at 15 pounds per square inch (psi). After autoclaving, 50 µg/ml of Ketoconazol was added to the medium, and allowed to cool to

about 45ºC, poured into sterile Petri-dishes and allowed to solidify. The medium was used to subculture of the isolates.

3.3.3 Potato dextrose agar (PDA)

Irish potato was peeled, and 40 g was weighed out, 200 ml of water was added, boiled with a bunsen burner, it was sieved, 1.6 g of dextrose was weighed out and 3 g of agar was also weighed. Dextrose and agar was diluted with the water from the boiled potato and make up to 200 ml. One hundred and ninety six milliliters (196ml) of the media was supplemented with 2 mM of phenol and sterilized by autoclaving at 121ºC for 15 mins. After sterilization, 50 µg/ml (10ml) of streptomycin was added to the medium, to inhibit the growth of bacteria. The medium was used for isolation of yeast isolates.

3.3.4 Mineral salt medium (MSM)

The mineral salt agar (NaCl, 10.0 g; MgSO4.7H_2O, 0.42 g; KCl, 0.29 g; KH_2PO4, 0.83 g; Na_2HPO4, 1.25 g; $NaNO_3$, 0.42 g; agar, 20 g; (Okpokwasili and Okorie, 1988) was dissolved in 1000 ml distilled water to make the MSM. The medium was sterilized by autoclaving at 121ºC for 15 mins at 15 pounds per square inch (psi) and the medium was allowed to cool. The medium was used for phenol utilization on plates.

3.3.5 Mineral salt broth (MSB)

The mineral salt agar (NaCl, 10.0 g; MgSO4.7H_2O, 0.42 g; KCl, 0.29 g; KH_2PO4, 0.83 g; Na_2HPO4, 1.25 g; $NaNO_3$, 0.42 g; (Okpokwasili and Okorie, 1988) was dissolved in 1000 ml distilled water to make the MSB. The medium was sterilized by autoclaving at 121ºC for 15 mins at 15 pounds per square inch (psi) and the medium was allowed to cool. The medium was used for phenol biodegradation assay.

3.3.6 Physiological saline

This was prepared by dissolving 0.85 g of sodium chloride in 100 ml of sterile distilled water. The solution was sterilization by autoclaving at 121ºC for 15 mins at 15 pounds per square inch (psi) and allowed to cool. This was used for serial dilution (Riquelme *et al.,* 2015).

3.4 Reagents

3.4.1 2, 3, 5-Triphenyltetrazolium chloride (TTC)

This was prepared by weighing out 0.1 g of TTC powder and dissolved in 20ml of water and made up to 100 ml in a volumetric flask (0.1%).This is artificial redox indicator used for the assessment of viability of microbial cells in the presence of toxicants.

3.5 Sample preparation and isolation

A 10 fold serial dilution of soil sample suspension was prepared by weighing out 10 g of soil samples into 90 ml of sterile physiological saline to form the stock. The mixture was stirred for 1 min in order to allow detachment of microbial cells from the soil particle. Using a sterile pipette, one militer (1.0 ml) of the stock was pipetted and transferred into a sterile test tube containing 9 ml of sterile physiological saline to obtain 10^{-1}dilution. 0ne milliliter (1 ml) was removed from the middle of the suspension and transferred into a 9 ml sterile physiological saline to achieve the 10^{-2} (always using a fresh sterile pipette for each transfer). The content of the 10^{-2} dilution test tube was shaken and dilution continued until the 10^{-8} dilution was obtained. Thereafter, an aliquot 0.1 ml of the dilutions $10^{-5,}$ $10^{-6,}$ and 10^{-7} were plated out in triplicates on potato dextrose agar (PDA) supplemented with 50 µg/ml of streptomycin antibiotic solution and 2 mM of phenol, nutrient agar supplemented with 50 µg/ml of ketoconazole, and nutrient agar plate without supplements respectively. The plates were then incubated at 30ºC for 24 h for bacteria and total heterotrophic count and 30ºC for 48 h for fungi count. The colonies formed on each plate were counted and the average counts were recorded as colony forming units per ml (cfu/g) of the soil sample.

3.6 Screening of isolates for degradation of 2, 3, 5 – triphenyltetrazolium chloride

After incubation, the best performing organisms were selected by adding a drop of 0.1% 2, 3, 5-triphenyltetrazolium chloride (TTC). The organisms that degraded TTC by turning red were selected (Nwanyanwu and Abu, 2012).

3.7 Purification of isolates

Two distinctive colonies that degraded TTC were picked and labeled as big round white (BRW) and small round white (SRW) respectively. They were subcultured onto PDA and the plates were incubated for 48h at 30ºC. The purified isolates were sub cultured into nutrient agar slant and stored in a refrigerator at 4ºC for identification and further test.

3.8 Identification of selected isolates

The selected fungi strains were identified based on morphological, microscopy and biochemical (Assimilation and Fermentation) characteristics according to Campbell *et al.,* (2013).

3.9 Phenol

The stock solution (100 mM) was prepared by dissolving 0.941 g of phenol into 50 ml of sterile distilled water and the volume was made up to 100 ml in a 100 ml volumetric flask. The solution was further diluted to obtain working stock concentration of 50 mM.

3.10 Growth on different concentrations of phenol amended mineral salt agar.

The mineral salt agar was prepared as described in section 3.3.4. The medium (50 ml) contained in 100 ml Erlenmeyer flasks was supplemented with different concentrations ranging from 0-6.0 mM of phenol respectively. The stock solution was prepared by dissolving 0.094 g of phenol in 100 ml of sterile distilled water. Thereafter, the flasks were sterilized by autoclaving at 121ºC for 20 min. after which the medium were poured into Petridish plates and allowed to solidify at room temperature (28±2) ºC. Then one tenth (0.1 ml) inoculum of the standardized organisms (OD_{600nm} 0.2) that was serially diluted to 10^{-3} was transferred onto the surface of the agar medium and spread plated. The plates were incubated at 30ºC for 72 h after which the colonies that developed were enumerated as colony forming unit per milliliter (cfu/ml).

3.11 Hydrocarbon tolerance of the fungi strains

The mineral salt broth was prepared as described in section 3.3.5. The hydrocarbon tolerance of the yeast was done in 5 ml volume of mineral salt broth medium (pH7.2) supplemented with 0.05 ml of different hydrocarbons (petrol, kerosene, diesel, lubricating oil, phenol, butanol and crude oil). A 4.0 ml portion of

mineral salt broth and 0.05 ml of different hydrocarbons were added to each 20 ml screw capped glass test tube. Thereafter, 1.0 ml of the standardized yeast cells (OD_{600nm} 0.1) was inoculated into each tube. The cultures were incubated at room temperature (28± 2ºC) for 7 days. After incubation, the tubes were visually examined and their optical densities (OD_{600nm}) were read off and recorded (Sandhu *et al.,* 2016).

3.12 Biodegradation of phenol

3.12.1 Standard calibration curve of phenol

The stock solution (1000 mg/l) was prepared by dissolving 0.1 g of phenol into 50 ml of sterile distilled water and the volume was made up to 100 ml in a 100 ml volumetric flask. From this, it was further diluted (2.5 ml of phenol solution above into 47.5 ml of water) to obtain working stock concentration of 50 mg/l. Thereafter, the solution was dispensed into test tubes accordingly according to Table 3.1 to obtain standard graded concentration of phenol. The absorbance of the standard solution was measured with a spectrophotometer (VIS spectrophotometer 721D, Life Assistance Scientific INST. CO) at 500 nm wavelength. The standard calibration curve of the phenol was determined by plotting absorbance verses concentrations. This was used as a standard phenol reference.

Table 3.1: Protocol for obtaining different concentrations used for phenol standard calibration curve

Tube	1	2	3	4	5	6	7	8
Stock 50 mg/l (ml)	0	0.025	0.05	0.1	0.2	0.4	0.6	0.8
Water (ml)	4.6	4.575	4.55	4.5	4.4	4.2	4.0	3.8
NH_4OH (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
4-Aminoantipyrine (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
$K_3Fe(CN)_6$ (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Concentration (ml)	0	0.25	0.5	1.0	2.0	4.0	6.0	8.0

3.12.2 Biodegradation assay

Degradation of phenol by the fungal species were carried out in sterile mineral salt broth as described in section 3.3.5, contained in 250 ml Erlenmeyer flasks. The flasks were supplemented with aliquot of sterile phenol (2000 mg/l) to bring the final phenol concentrations in the flasks to 100, 200, 500, 750 and 1000 mg/l. Thereafter, 2 ml of standardized (OD_{600nm} 0.4) cell suspension was added. The cultures were incubated at room temperature (28 ± 2°C) on a rotary shaker operated at 150 rpm. Samples were withdrawn periodically (24 h) to monitor the growth profile and utilization of phenol by the isolates. Phenol residue was determined in cell free supernatant via 4-aminoantipyrine technique (Nwanyanwu and Abu, 2012).

3.12.2.1Measurement of growth of pure cultures

Growth profile of the test organisms was monitored by using optical density measurement (Nwanyanwu and Abu, 2012). Four milliliters (4 ml) of the sample was drawn periodically (24 h) and optical densities (A_{600}) of the cultures were determined spectrophotometrically

3.12.2.2 Assay for phenol residue

Phenol residue of the fungal culture was determined using 4-aminoantipyrine method based on the procedure as described by Nwanyanwu and Abu. (2012). Culture samples were withdrawn and centrifuged 6000 rpm for 10 min to remove the cells. Into 4 ml of the culture supernatant withdrawn contained in 20 ml screw capped glass test tubes, 0.2 ml of 0.5 N NH_4OH was added, followed by addition of 0.1 ml 2 %(w/v) 4-aminoantipyrine and 0.1 ml of 8 %(w/v) potassium ferricyanide [$K_3Fe(CN)_6$] and the contents thoroughly mixed. The absorbance (A_{500}) of the resultant solution was determined spectophotometrically. Phenol residue was then calculated by making reference to the standard curve in appendix 11.

3.12.2.3. Specific growth rate

The specific growth rates (μ, h^{-1}) were taken from $\ln(X/X_0)$ versus $t-t_0$ plots for each initial phenol concentration. In each case, values were taken as the maximum slope in the respective plots (Nweke and Okpokwasili, 2014).

$$\ln\frac{X}{X_0} = \mu(t - t_0)$$ **Equation 1**

3.12.2.4 Biodegradation rate

Phenol biodegradation rate (Qs, mg/l.h) was determined through the relationship of equation 2 (Nwanyanwu and Abu, 2012).

$$\mathbf{Q_s} = \frac{\mathbf{d[ph]}}{\mathbf{dt}} \qquad \text{Equation 2}$$

Where: [Ph] denotes phenol concentration (mg/l), t denotes incubation time (h).

3.12.2.5 Specific degradation rate

The specific degradation rate (Qs, mg/l.(h.OD)) was determined through the relationship of equation 3 (Nwanyanwu and Abu, 2013).

$$\mathbf{Q_s} = \frac{\mathbf{d[ph]/dt}}{\mathbf{X}} \qquad \text{Equation 3}$$

Where: [Ph] denotes phenol concentration (mg/l), t denotes incubation time (h) and X denotes cell concentration (Optical density, OD_{600} nm).

3.13 Toxicity assay of phenol and two heavy metal mixtures

3.13.1 Test chemicals and their preparations:

The two heavy metal used in the assay are zinc (Zn^{+2}) used as $ZnSO_4$.$7H_2O$ and cadmium (Cd^{+2}), used as $CdSO_4$. Phenol used was obtained from Sigma-chemical Co. USA,

3.13.2 Zinc ion (Zn^{+2}) and cadmium ion (Cd^{+2})

The stock solution (10 mM) of zinc and cadmium were prepared by dissolving 0.2875 g and 0.2565 g of the salt in 50 ml of sterile distilled water respectively and the volumes were made up to 100 ml in a 200 ml volumetric flasks (Nwanyanwu *et al.*, 2017).

3.13.3 Phenol

The stock solution (100 mM) was prepared by dissolving 0.941g of phenol into 50 ml of sterile distilled water and the volume was made up to 100 ml in a 200 ml volumetric flask. From this, it was further diluted with 100ml of sterile distilled water to obtain working stock concentration of 50 mM (Nwanyanwu *et al.*, 2017).

3.13.4 Preparation of inoculum

The isolates were grown in sterile nutrient broth medium and incubated on a rotary shaker (150 rpm) incubator for 48 h at room temperature (28 ± 2ºC) .Thereafter, the cells were harvested by centrifugation (ALPIN MEDICAL ENGLAND90 (1)) at 6000 rpm for 10 mins.The harvested cells were washed twice in sterile distilled water to ensure that there are no leftover of nutrient broth. The washed cells were suspended in nutrient broth and the optical density (OD) adjusted to 0.2 at wavelength of 600 nm using spectrophotometer (VIS spectrophotometer 721D, Life Assistance Scientific INST. CO) (Nwanyanwu *et al*., 2017).

3.13.5 Enzyme assay in the presence of chemicals

Dehydrogenase activity inhibition was determined using 2, 3, 5-triphenyltetrazolium chloride (TTC) as the artificial electron acceptor which is reduced to the red-coloured triphenylformazan (TPF). The inhibition of dehydrogenase activity was done in 2 ml volume of nutrient broth (pH 7) and TTC supplemented with varying concentrations of zinc, phenol and cadmium as shown in Tables 3.2, 3.3 and 3.4 respectively. A 0.5 ml portion of x4-strength (0.2%) nutrient broth and required volumes of sterile distilled water and stock solution of the respective toxicants were added to each 20 ml screw capped glass test tube to obtain the different binary and ternary mixture ratios. Thereafter, 0.2 ml each of 0.1%w/v solution of TTC and yeast suspension was added into each tube. The final concentration of toxicants ranged from 0 to 5 mM for zinc, 0 to 16 mM for phenol and 0 to 0.4 mM for cadmium as individual chemical. Binary mixtures of zinc and cadmium ranged from 0 to 5.0 mM, phenol and zinc ranged from 0 to 16 mM, as shown in table 3.5 and 3.6 respectively. The ternary mixtures of zinc, phenol and cadmium ranged from to 0 to 16 mM as shown in table 3.7. The controls consist of the medium without the toxicants. The cultures were incubated at room temperature (28± 2ºC) for 24 h. After incubation, the formazan produced was extracted with 1 ml of 1%v/v triton x100 and 4 ml of butanol. Absorbance of the extract was determined spectrophotometrically at 500 nm (Nwanyanwu *et al*., 2017).

Table 3.2: Protocol for obtaining different concentrations used in the assessment of zinc as individual chemical toxicity to phenol-utilizing yeast strains

Tubes	1	2	3	4	5	6	7	8	9	10	11
Toxicant (ml)	0	0.04	0.1	0.16	0.2	0.28	0.4	0.5	0.6	0.8	1.0
Water (ml)	1.1	1.08	1.00	0.94	0.90	0.82	0.7	0.6	0.5	0.2	0.1
TTC (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Innoculum(ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Final conc (mM)	0	0.2	0.5	0.8	1.0	1.4	2.0	2.5	3.0	4.0	5.0

Table 3.3: Protocol for obtaining different concentrations used in the assessment of cadmium as individual chemical toxicity to phenol-utilizing yeast strains

Tubes	1	2	3	4	5	6	7	8	9	10	11
Toxicant (ml)	0	0.01	0.02	0.028	0.036	0.04	0.044	0.05	0.056	0.06	0.08
Water (ml)	1.1	1.08	1.00	0.94	0.9	0.82	0.7	0.6	0.5	0.4	0.2
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
TTC (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Innoculum (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Final conc(mM)	0	0.05	0.1	0.14	0.18	0.2	0.22	0.25	0.28	0.3	0.4

Table 3.4: Protocol for obtaining different concentrations used in the assessment of phenol as individual chemical toxicity to phenol-utilizing yeast strains

Tubes	1	2	3	4	5	6	7	8	9	10	11
Toxicant (ml)	0	0.02	0.04	0.08	0.1	0.12	0.16	0.24	0.4	0.48	0.64
Water (ml)	1.1	1.08	1.06	1.02	1.0	0.98	0.94	0.86	0.7	0.62	0.46
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
TTC (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Innoculum (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Final conc(mM)	0	0.5	1.0	2.0	2.5	3.0	4.0	6.0	10	12	16

Table 3.5: Protocol for obtaining different concentrations used in the assessment of binary mixtures of zinc and cadmium toxicity to phenol-utilizing yeast strains

Tubes	1	2	3	4	5	6	7	8	9	10	11
Toxicant (ml)	0	0.04	0.1	0.16	0.2	0.28	0.4	0.5	0.6	0.9	1.0
Water (ml)	1.1	1.08	1.00	0.94	0.9	0.82	0.7	0.6	0.5	0.4	0.2
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
TTC (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Innoculum (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Final conc(mM)	0	0.2	0.5	0.8	1.0	1.4	2.0	2.5	3.0	3.5	4.0

Table 3.6: Protocol for obtaining different concentrations used in the assessment of binary mixtures of zinc and phenol toxicity to phenol-utilizing yeast strains

Tubes	1	2	3	4	5	6	7	8	9	10	11
Toxicant (ml)	0	0.02	0.04	0.08	0.1	0.12	0.16	0.24	0.4	0.48	0.64
Water (ml)	1.1	1.08	1.06	1.02	1.0	0.98	0.94	0.86	0.7	0.62	0.46
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
TTC (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Innoculum (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Final conc(mM)	0	0.5	1.0	2.0	2.5	3.0	4.0	6.0	10	12	16

Table 3.7: Protocol for obtaining different concentrations used in the assessment of ternary mixtures of zinc and cadmium toxicity to phenol-utilizing yeast strains

Tubes	1	2	3	4	5	6	7	8	9	10	11
Toxicant (ml)	0	0.02	0.04	0.08	0.1	0.12	0.16	0.24	0.4	0.48	0.64
Water (ml)	1.1	1.08	1.06	1.02	1.0	0.98	0.94	0.86	0.7	0.62	0.46
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
TTC (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Innoculum (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Final conc(mM)	0	0.5	1.0	2.0	2.5	3.0	4.0	6.0	10	12	16

3.13.6 Design of experiment for binary and ternary mixtures

The toxicity of assessment of the binary and ternary mixtures of the chemicals on the dehydrogenase activity of phenol utilizing yeasts was studied using uniform ratio and equi-concentration effect as shown in Tables 3.5, 3.6 and 3.7 respectively. The binary mixture ratio of zinc and cadmium evaluated were 66.7% zinc + 33.3% cadmium, 88.8% zinc +11.2% cadmium, 93.3% zinc + 7.7% cadmium and 75% zinc + 25% cadmium. The binary mixture ratio of zinc and phenol were 5.9% zinc + 94.1% phenol, 20% zinc + 80% phenol, 27.3% zinc +72.7% phenol and 50% zinc + 50% phenol. Ternary mixture ratio of zinc, phenol and cadmium were, 12.9% zinc +86.2% phenol +0.9% cadmium, 27.2% zinc + 71.1% phenol +1.7% cadmium and 28% zinc + 70% phenol + 2% cadmium. The equi effect concentration studied was equi-effect concentration 50% (EE_{50}) that is the concentration that inhibited 50% of the yeast enzyme activity (Nwanyanwu *et al*., 2017).

3.13.7. Estimation of response of organisms to effect of the chemicals

The inhibition of dehydrogenase activity at varying concentrations of the individual component, binary and ternary mixtures of phenol, cadmium and zinc were calculated relative to the control using the expression

$$\%INH = \frac{C_A - T_A}{C_A} \times 100$$

C_A is the absorbance of TPF extract in the control

T_A is the absorbance of TPF extract in the test with different concentrations of the toxicants as single, binary and ternary mixtures.

3.13.8 Determination of toxicity threshold of phenol, zinc and cadmium on yeast strains

The dose response data from the assessment of toxic effects of the toxicants in their binary and ternary mixtures on dehydrogenase activities of the yeast were tested with 3-parameter logistic model to obtain their respective toxicity threshold (IC_{50}) which is defined as the concentrations of the toxicants that inhibited the dehydrogenase activity of the yeast by 50% (Nwanyanwu *et al*., 2017).

$$\% INH = \frac{100}{1 + \left(\frac{x}{IC_{50}} \right)^{b}}$$

3.12.9 Analysis of combined effects using toxic index model

Toxic index model (TI) was used to analyze the combined effect of the binary and ternary mixtures.

The TI values of binary were calculated using the expression:

$$TI = \frac{C_{mixA}}{IC_{50A}} + \frac{C_{mixB}}{IC_{50B}}$$

$$C_{mixA} = \frac{A\%}{100} \times IC_{50mix(A,B)}$$

$$C_{mixB} = \frac{B\%}{100} \times IC_{50mix(A,B)}$$

$$TI = \frac{C_{mixB}}{IC_{50B}} + \frac{C_{mixC}}{IC_{50C}}$$

$$C_{mixA} = \frac{B\%}{100} \times IC_{50mix(B,C)}$$

$$C_{mixB} = \frac{C\%}{100} \times IC_{50mix(B,C)}$$

The TI values of ternary were calculated using the expression:

$$\mathbf{TI} = \frac{\mathbf{C_{mixA}}}{\mathbf{IC_{50A}}} + \frac{\mathbf{C_{mixB}}}{\mathbf{IC_{50B}}} + \frac{\mathbf{C_{mixC}}}{\mathbf{IC_{50C}}}$$

$$C_{mixA} = \frac{A\%}{100} \times IC_{50mix(A,B,C)}$$

$$C_{mixB} = \frac{B\%}{100} \times IC_{50mix(A,B,C)}$$

$$C_{mixC} = \frac{C\%}{100} \times IC_{50mix(A,B,C)}$$

Where A%, B% and C% are the relative amount of components A, B and C respectively in the mixtures (A%, B% and C% $= 0$).

When A =0%, C_{mixA}=0, C_{mixB}=IC_{50B} and C_{mixC}=IC_{50C}.

When B =0%, C_{mixB}=0, C_{mixC}=IC_{50C} and C_{mixA}=IC_{50A}.

When C =0%, C_{mixC}=0, C_{mixA}=IC_{50A} and C_{mixB}=IC_{50B}.

C_{mixA}, C_{mixB} and C_{mixC} are the concentrations of component A, B and C respectively at the IC_{50} of the mixture; IC_{50A}, IC_{50B} and IC_{50C} are the IC_{50} of the component A, B and C respectively, measured individually.

TI=1 describes additive interaction, TI>1 describes antagonistic interaction and TI<1 describes synergistic interaction (Boillot and Perrodin, 2008)

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 RESULTS

4.1.1 Identification of isolates

The result of the physicochemical and microbiological analysis of hydrocarbon impacted soil is shown in Table 4.1. Identification of the isolates based on their morphology, microscopic and biochemical characteristics (Assimilation and fermentation reactions) are shown in Tables 4.2 and 4.3 respectively. The isolates identified were *Cryptococcus* sp and *Saprochaete* sp.

4.1.2 Growth of yeast strains on different concentrations of phenol amended mineral salt agar.

Figure 4.1 shows growth response of the yeasts to increase doses of phenol on mineral salt medium supplemented with different phenol concentrations ranging from 0-6 mM. There was a decrease in the number of the yeasts as the concentration of the phenol increases.

Table 4.1: Physicochemical and microbiological analyses of the hydrocarbon impacted soil sample.

Parameter / unit	Value
Ph	6.88
Phosphate (mg/kg)	18.64
Potassium (cmol/kg)	0.076
Calcium (cmol/kg)	0.986
Magnesium (cmol/kg)	0.372
Copper (kg)	0.50
Zinc (kg)	1.56
Lead (kg)	8.90
Cadmium (kg)	3.70
Total hydrocarbon content (%)	0.465
Total organic carbon content (%)	1.246
Total organic matter content (%)	2.148
Total nitrate content (%)	0.120
Soil texture (%)	
Silt	6.80
Clay	5.96
Sand	87.24
Microbiological analysis	
Microbial heterotrophic count (cfu/g)	3.3×10^6
Bacteral count (cfu/g)	1.6×10^5
Phenol-utilizing yeast count (cfu/g)	6.0×10^4

Table 4.2: Colonial, morphological and microscopic characteristics of the isolates

Isolate	Shape	Colour	Appearance	Blastospore	Budding	Capsule	Hyphae
BRW	Oval	Brown	Smooth	P	p	P	A
SRW	Round	White	Heaped	A	P	A	P

Key: P = Presence, A = Absent, BRW = Big round white, SRW = Small round white

Table 4.3: Biochemical characteristics of the isolates based on assimilation and fermentation reactions

Isolate	Glucose	Maltose	Sucrose	Lactose	Rafinose	Trehalose	Galactose	Manitol	Rhamnose	Cellulose	Nitrate	Urease	Identity of isolate
Assimilation reaction													
A	+	-	-	-	-	+	+	+	+	+	N	N	*Cryptococcus* sp
B	+	-	-	-	-	-	+	+	-	-	N	N	*Saprochaete* sp
Fermentation reaction													
A	-	-	-	-	N	-	N	N	N	N	-	+	*Cryptococcus* sp
B	-	-	-	-	N	-	N	N	N	N	-	-	*Saprochaete* sp

Key: + = Positive, - = Negative, N = Not determined

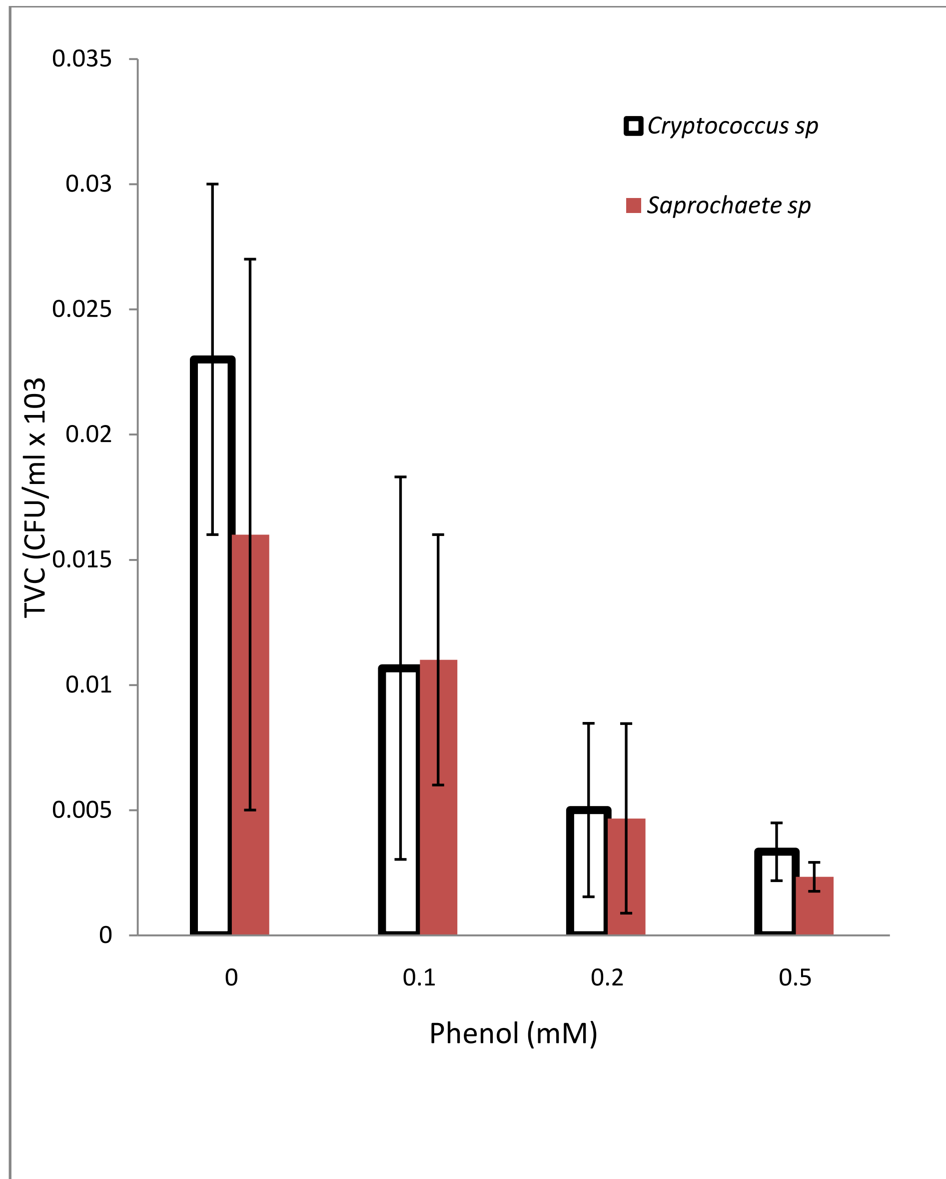


Figure 4.1: Growth response of the yeasts to increase doses of phenol in MSM.

4.1.3 Hydrocarbon tolerance of the fungal strains

Table 4.4 shows the hydrocarbon tolerance of the isolates. Both *Saprochaete* sp and *Cryptococcus* sp showed heavy growth on diesel and crude oil. *Saprochaete* sp exerted the lowest growth on petrol, kerosene and butanol, while for *Cryptococcus* sp there was no growth on petrol, lubricating oil and butanol. *Saprochaete* sp recorded moderate growth for lubricating oil and phenol, while *Cryptococcus* sp recorded low growth on kerosene and phenol. Table 4.5 shows the optical reading of optical densities of the hydrocarbon tolerance of the yeast strains on mineral salt broth. The highest optical density was recorded at crude oil (0.109) for *Saprochaete* sp, while for *Cryptococcus* sp; the highest optical density was recorded at diesel (0.1775). The lowest optical density was recorded at petrol (0.0225) for *Saprochaete* sp, while for *Cryptococcus* sp; the lowest optical density was recorded at lubricating oil (0.0185).

Table 4.4: Hydrocarbon Tolerance of the isolates

Hydrocarbon substrate	Organisms	
	***Saprochaete* sp**	***Cryptococcus* sp**
Crude oil	+++	+++
Lubricating oil	++	-
Diesel	+++	+++
Petrol	+	-
Kerosene	+	+
Butanol	+	+
Phenol	++	+

Key: +++ = Heavy growth, ++ = Moderate growth, + = growth, - = No growth

Table 4.5: Optical densities of the hydrocarbon tolerance of the yeasts on MSB

	Optical density	Optical density
Hydrocarbons	***Saprochaete* sp**	***Cryptococcus* sp**
Petrol	0.0225	0.0205
Kerosene	0.0245	0.055
Diesel	0.0945	0.1775
Phenol	0.0365	0.0415
Butanol	0.029	0.0225
Lubricating oil	0.061	0.0185
Crude oil	0.109	0.168

Key: MSB= Mineral salt broth medium

4.1.4 Phenol degradation by the yeast strains

Figure 4.2 shows phenol degradation at different initial concentrations by the yeasts, and figure 4.3 shows the growth profile of the yeast cultures containing different concentrations of phenol. The phenol in the mineral salt broth (MSB) was completely degraded by the organisms within 3 (72 h) to 14 (336 h) days of incubation. One hundred milligrams per litre (100 mg/l) and seven hundred and fifty milligrams per liter (750 mg/l) of phenol were degraded completely within three days [3 d (72 h)] and ten days [10 d (240 h)] of incubation by both yeasts. For one thousand milligrams per litre (1000 mg/l) of phenol, *Saprochaete* sp degraded the phenol within fourteen days [14 d (336 h)] of incubation, while *Cryptococcus* sp degraded the phenol within eleven days [11 d (264 h)] of incubation.

Table 4.5 shows the biodegradation responses of the isolates. The specific growth rate of both yeasts decreases as the initial phenol concentrations were increased. The highest specific growth rate was recorded at 100 mg/l with 0.030 h^{-1} and 0.037 h^{-1} for *Saprochaete* sp and *Cryptococcus* sp respectively; while the lowest specific growth rate was recorded at 750 ml/l and 1000 mg/l with the same value (0.009 h^{-1}) for *Cryptococcus* sp and 0. 005 h^{-1} *Saprochaete s*p. The rates of degradation of both organisms are the same at phenol concentration of 100 mg/l and 500 mg/l with 0.083 mg/l.h and 0.250 mg/l.h respectively. Both yeasts recorded the highest degradation rate at phenol concentration of 500 mg/l with 0.250 mg/l.h and lowest rate at phenol concentration of 100 mg/l with 0.083 mg/l.h. *Saprochaete* sp and *Cryptococcus* sp recorded the highest specific degradation rate at phenol concentration of 500 mg/l with 2.137 mg/l.h.OD and 1.639 mg/l.h.OD respectively, while the lowest specific degradation rate was recorded at phenol concentration of 1000 mg/l with 0.707 mg/l.h.OD for *Saprochaete* sp and *Cryptococcus* sp recorded 0.582 mg/l/h/OD at 750 mg/l.

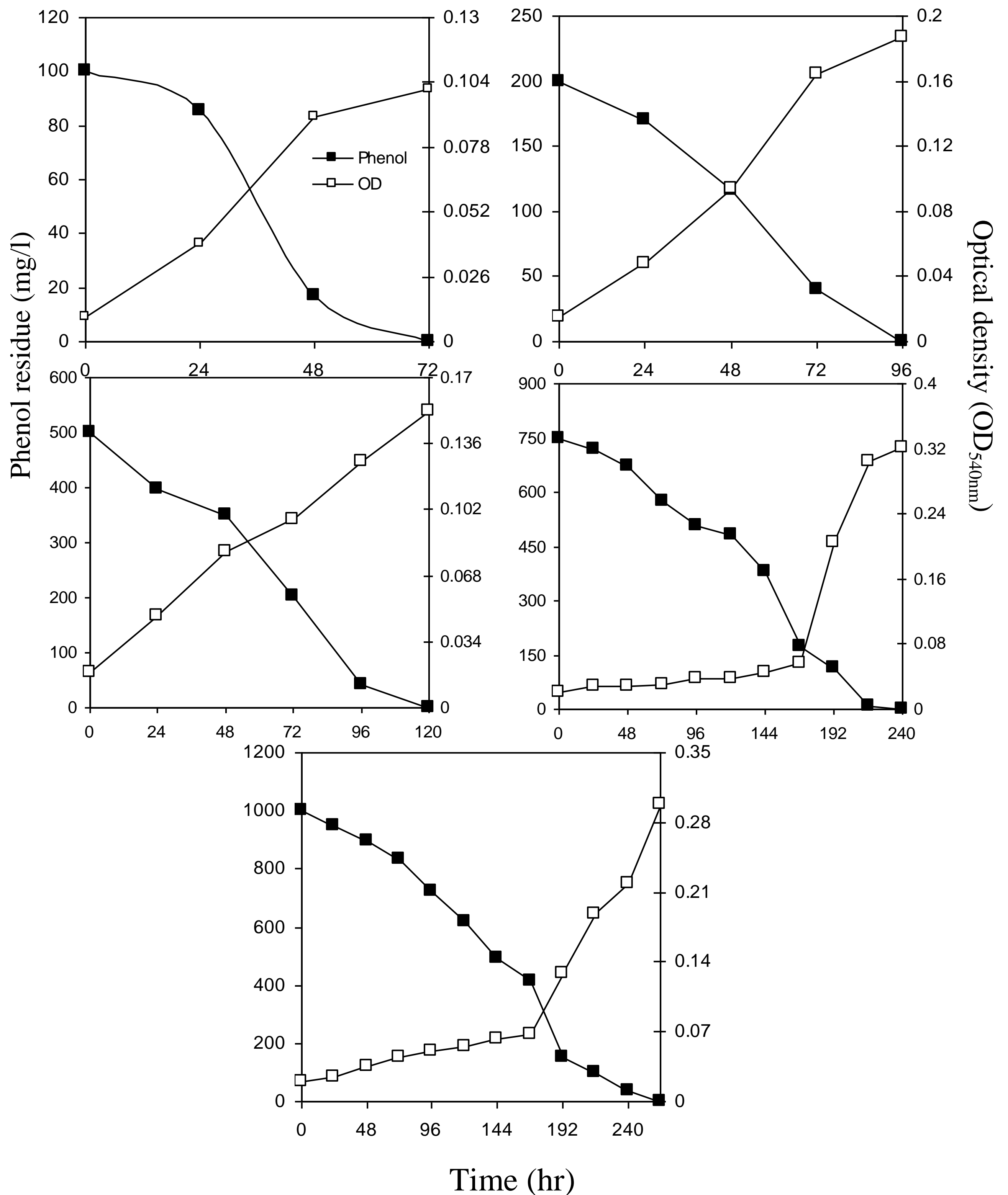


Figure 4.2: Growth profile and phenol degradation at different concentrations by *Cryptococcus* sp

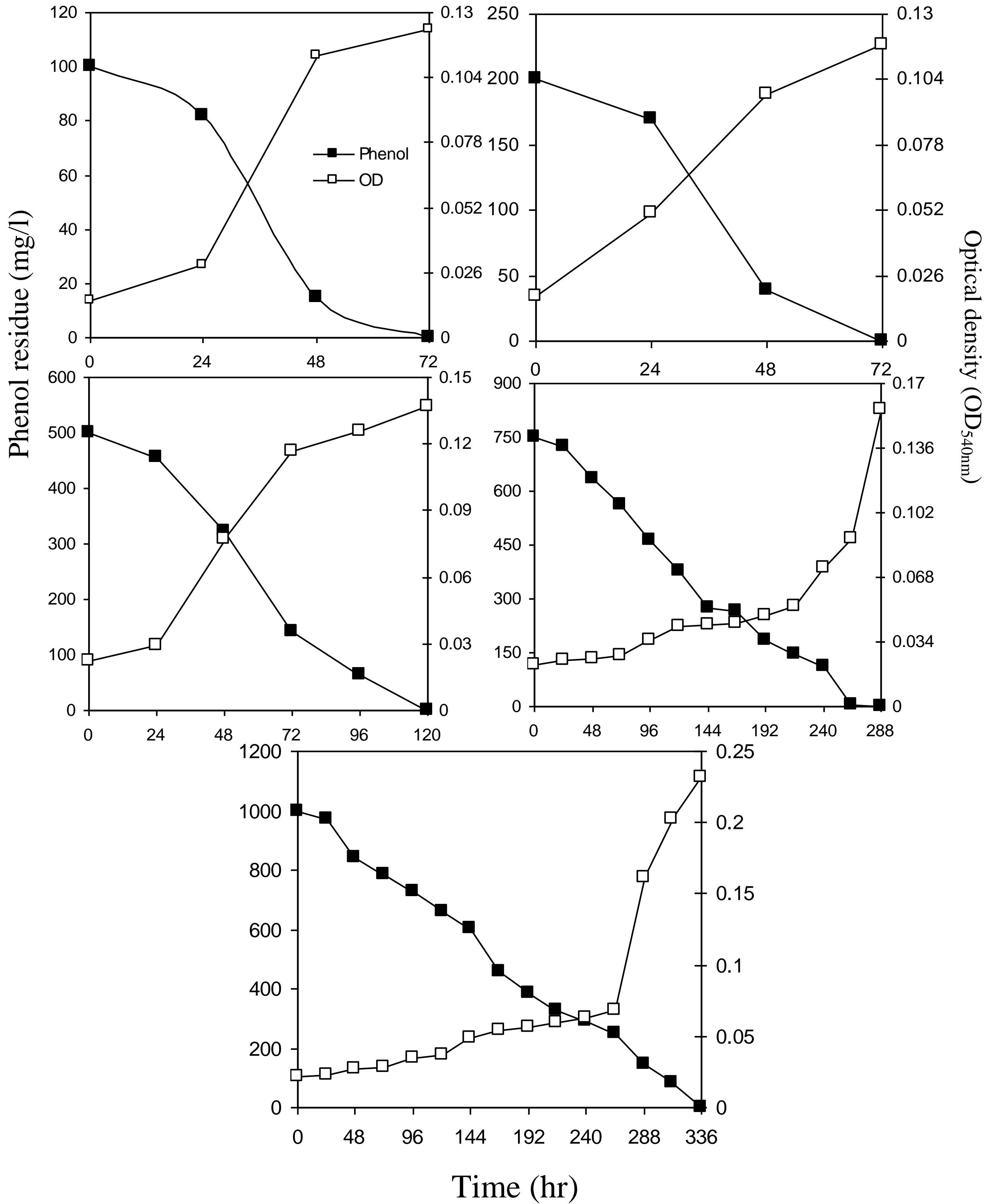


Figure 4.3: Growth profile and phenol degradation at different concentrations by *Saprochaete* sp

Table 4.6: Biodegradation response of the yeast isolates

Phenol concentration (mg/l)	Organism	
	Cryptococcus sp	*Saprochaete* sp
	Specific growth rate (h^{-1})	
100	0.037	0.034
200	0.030	0.025
500	0.020	0.016
750	0.009	0.005
1000	0.009	0.005
	Biodegradation rate (mg/l.h)	
100	0.083	0.083
200	0.125	0.167
500	0.250	0.250
750	0.188	0.156
1000	0.227	0.164
	Specific biodegradation rate (mg/l.(h.OD))	
100	0.825	0.667
200	0.668	1.984
500	1.639	2.137
750	0.582	1.002
1000	0.764	0.707

4.1.5 Toxicity of single chemicals to the yeast strains

The toxicity of zinc, phenol and cadmium as single chemical on *Saprochaete* sp. are shown in Figures 4.4, 4.5 and 4.6, while Figure 4.7, 4.8 and 4.9 showed the toxicity of zinc, phenol and cadmium as single chemical on *Cryptococcus* sp. Phenol showed hormetric (stimulatory) effect on the enzyme activities of the yeast and progressively inhibited the enzyme activity, cadmium exhibited sharp inhibitory effect as the concentration increases, and zinc showed a progressive inhibition of the dehydrogenase activity of both yeasts as the concentration increases.

4.1.6 Toxicity of binary mixtures of chemicals to the yeast strains

Figure 4.10, 4.11, 4.12, 4.13, 4.14, 4.15, 4.16 and 4.17 shows the model fit curves for the assessment of toxic effects of binary mixtures of zinc and phenol on phenol-utilising yeasts. Figure 4.18, 4.19, 4.20, 4.21, 4.22, 4.23, 4.24 and 4.25 shows the model fit curves for the assessment of toxic effects of binary mixtures of zinc and cadmium on phenol-utilizing yeasts. The binary mixture ratio of 27.3% zinc and 72.7% phenol and 50% zinc and 50% phenol shows inhibitory effects on the enzyme activity as the concentration increases, mixtures ratios of 5.9% zinc + 94.1% phenol and 20% zinc + 80% phenol respectively showed stimulatory effects at low concentrations for dehydrogenase activity of *Saprochaete* sp. Above the hermetic concentration range, the chemicals inhibited the dehydrogenase activites of the yeasts. The binary mixtures of zinc and cadmium showed progressive inhibitory effects on the dehydrogenase (enzyme) activity of the test yeast as the concentration increases. No stimulatory effects were observed.

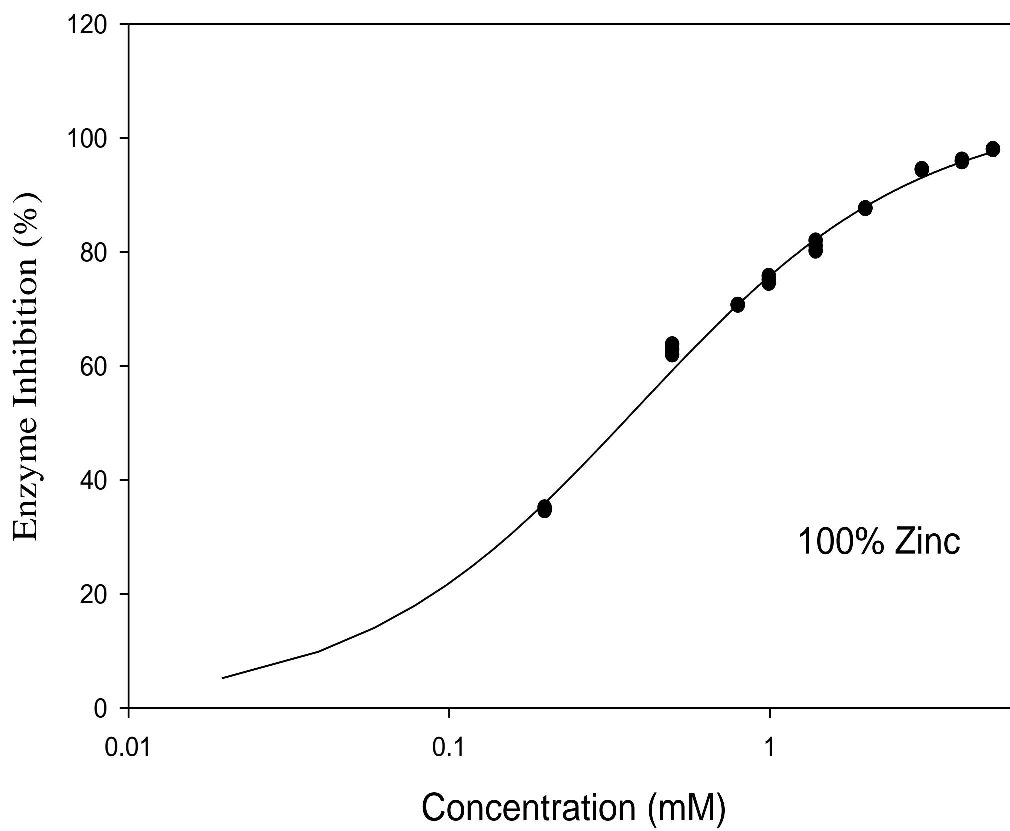


Figure 4.4: Toxicity of zinc as single chemical on *Saprochaete* sp.

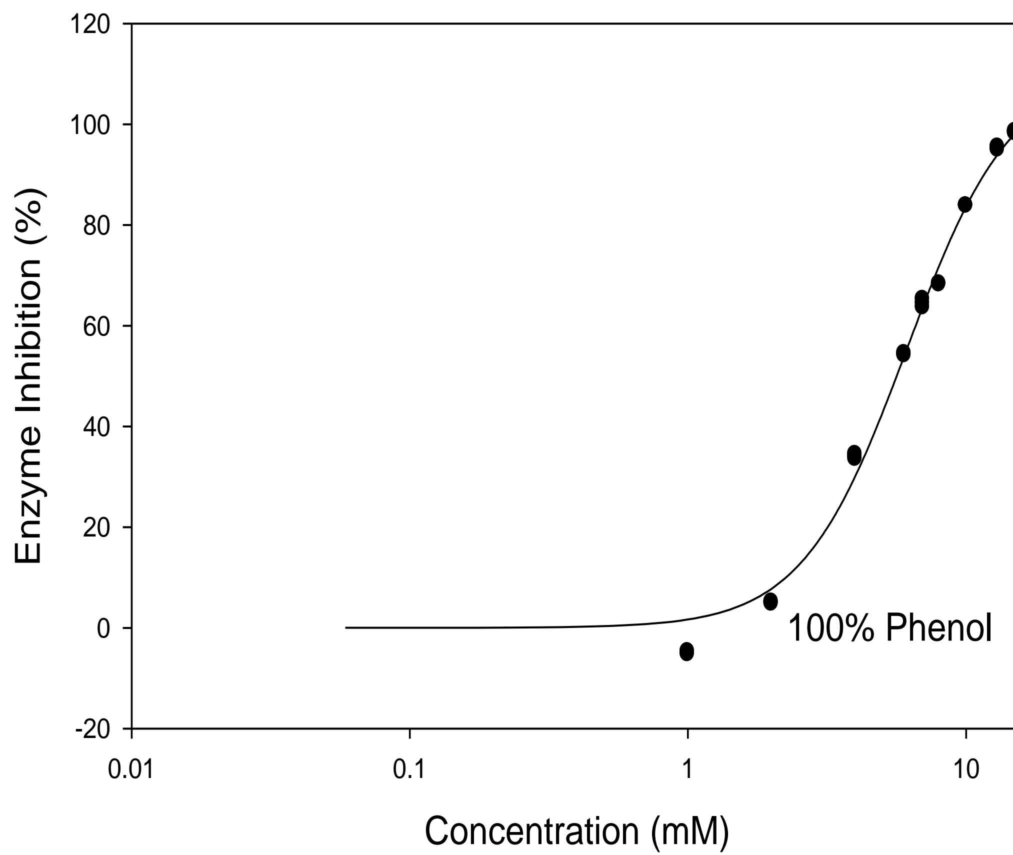


Figure 4.5: Toxicity of phenol as single chemical on *Saprochaete* sp.

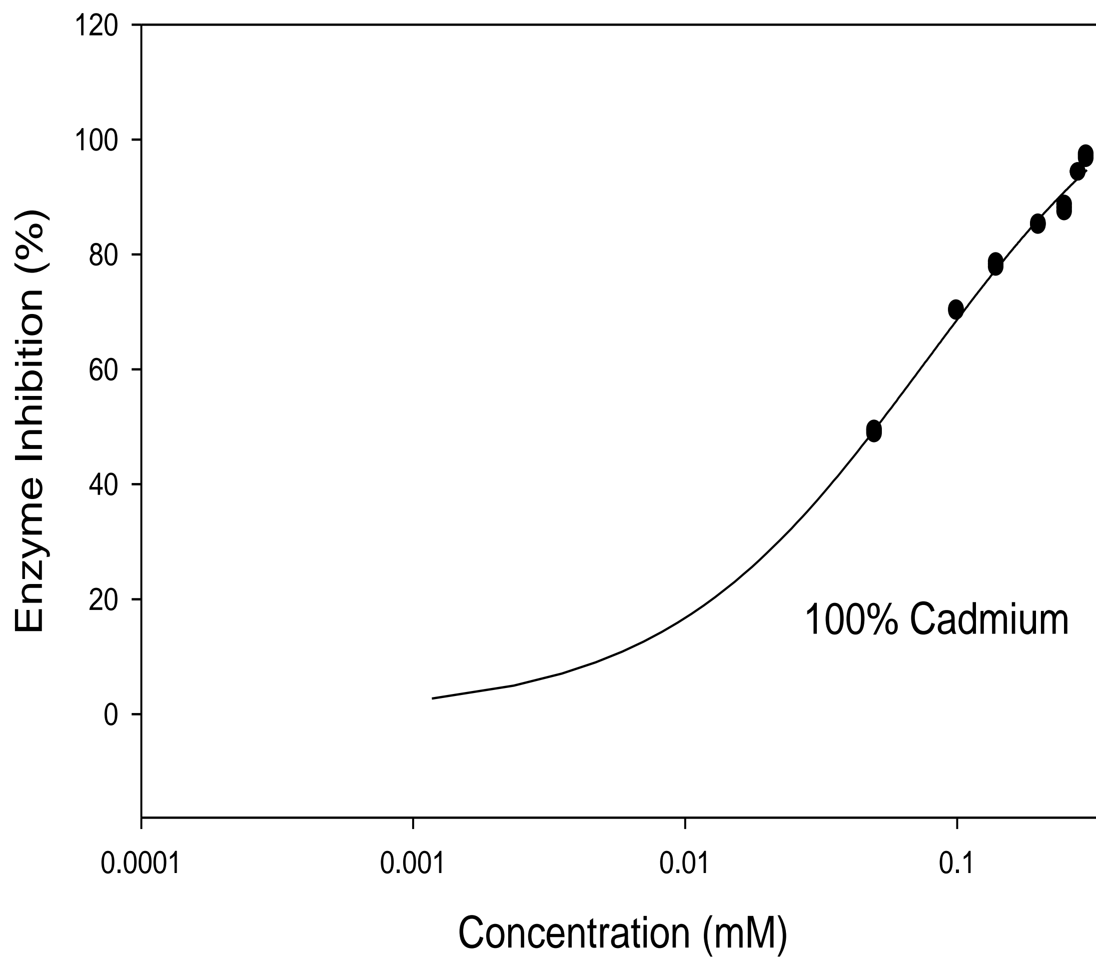


Figure 4.6: Toxicity of cadmium as single chemical on *Saprochaete* sp.

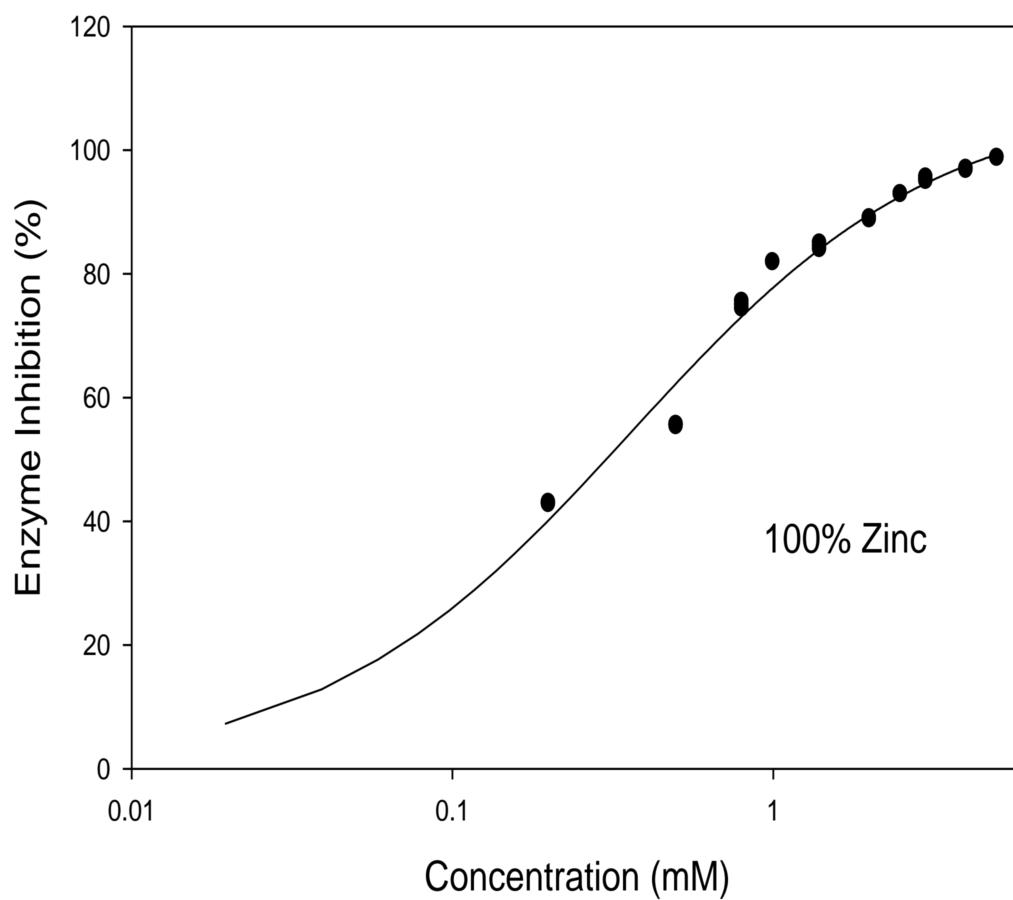


Figure 4.7: Toxicity of zinc as single chemical on *Cryptococcus* sp.

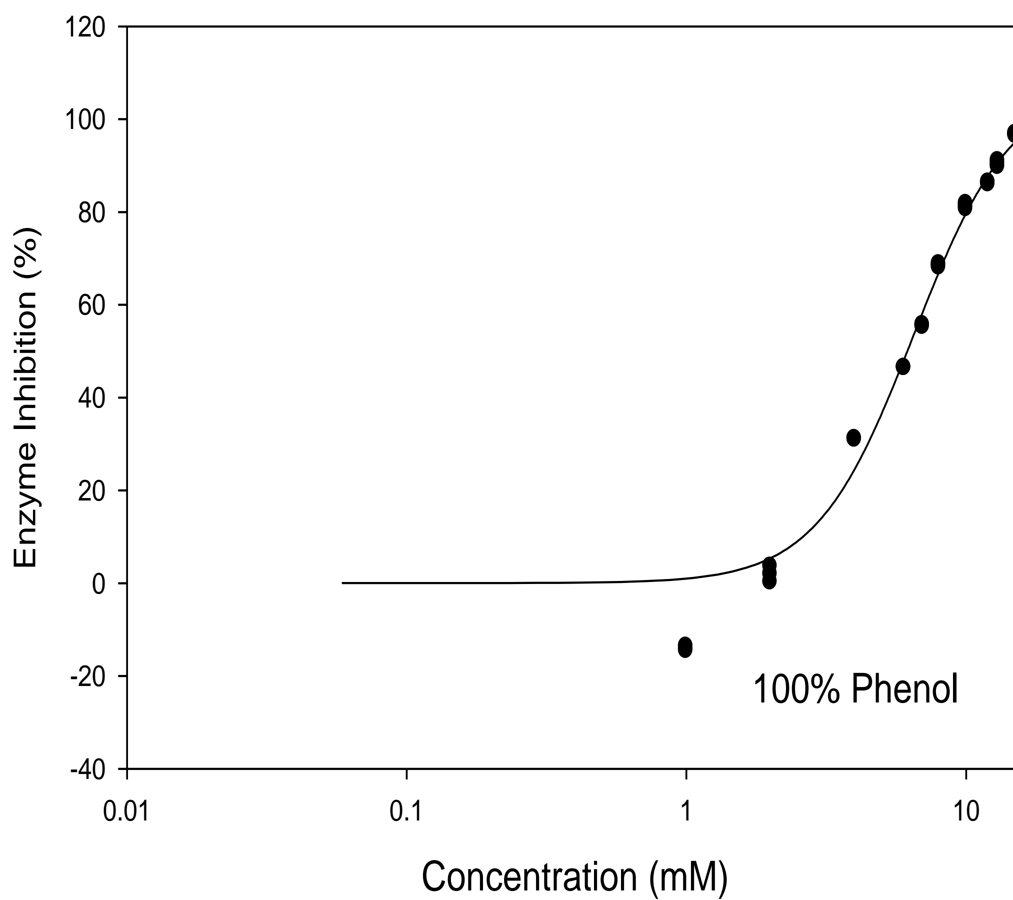


Figure 4.8: Toxicity of phenol as single chemical on *Cryptococcus sp.*

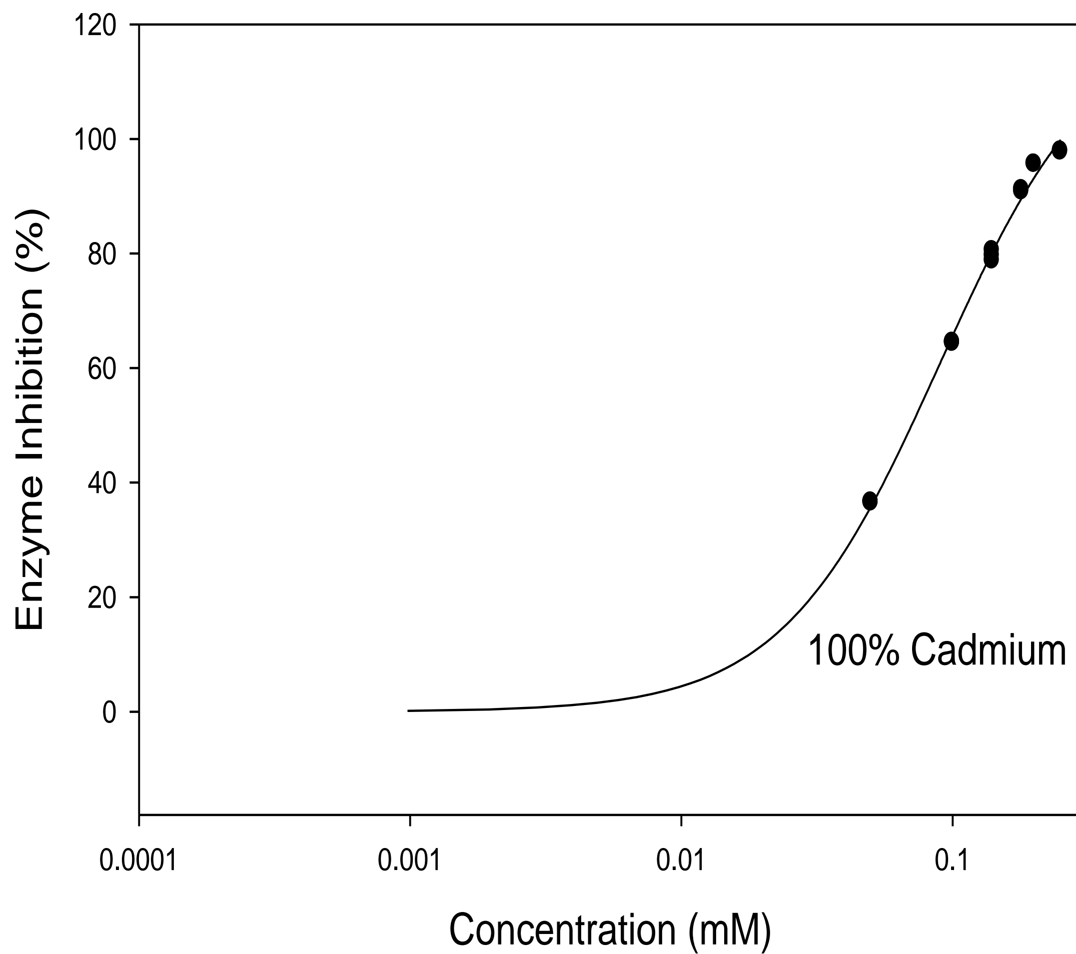


Figure 4.9: Toxicity of cadmium as single chemical on *Cryptococcus* sp.

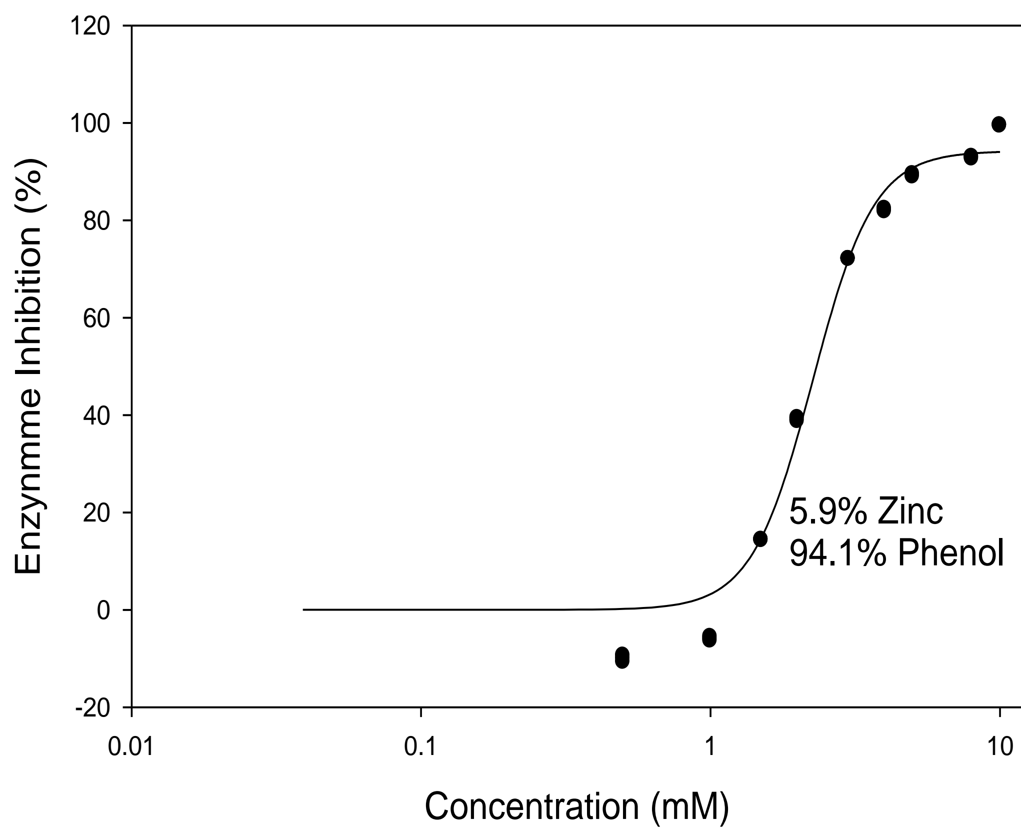


Figure 4.10: Toxicity of binary mixture of zinc and phenol on *Saprochaete* sp.

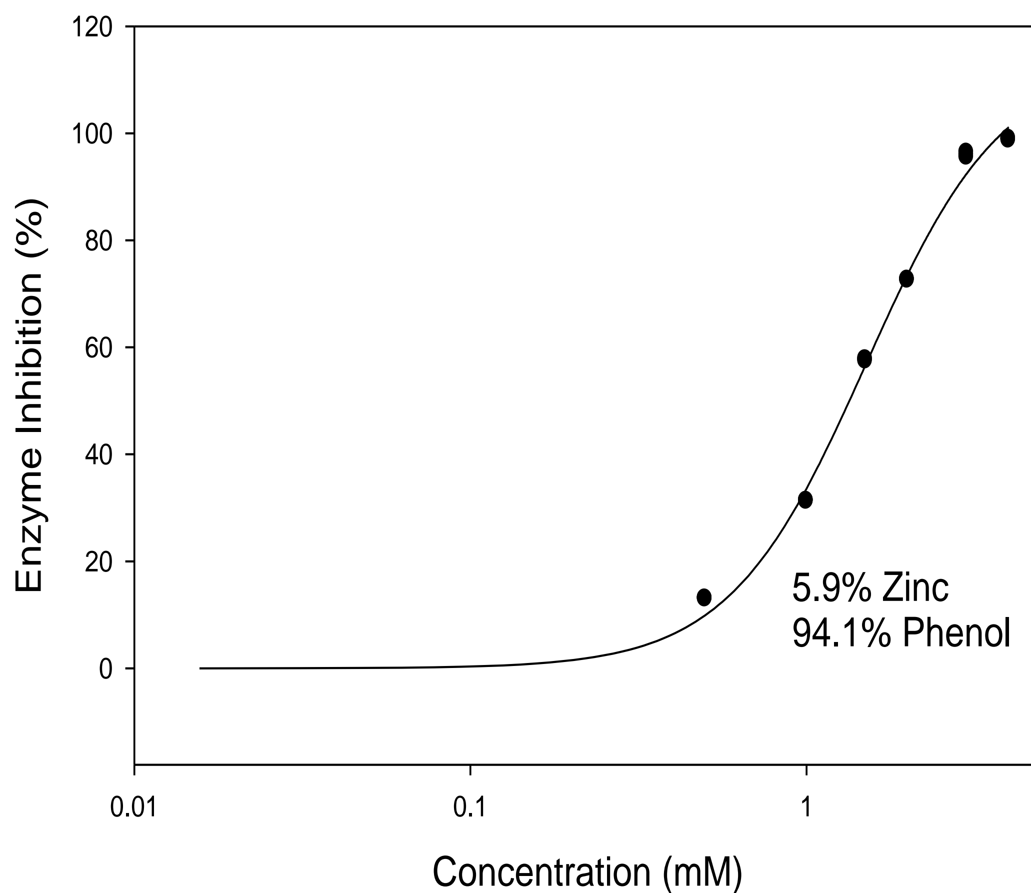


Figure 4.11: Toxicity of binary mixture of zinc and phenol on *Cryptococcus* sp.

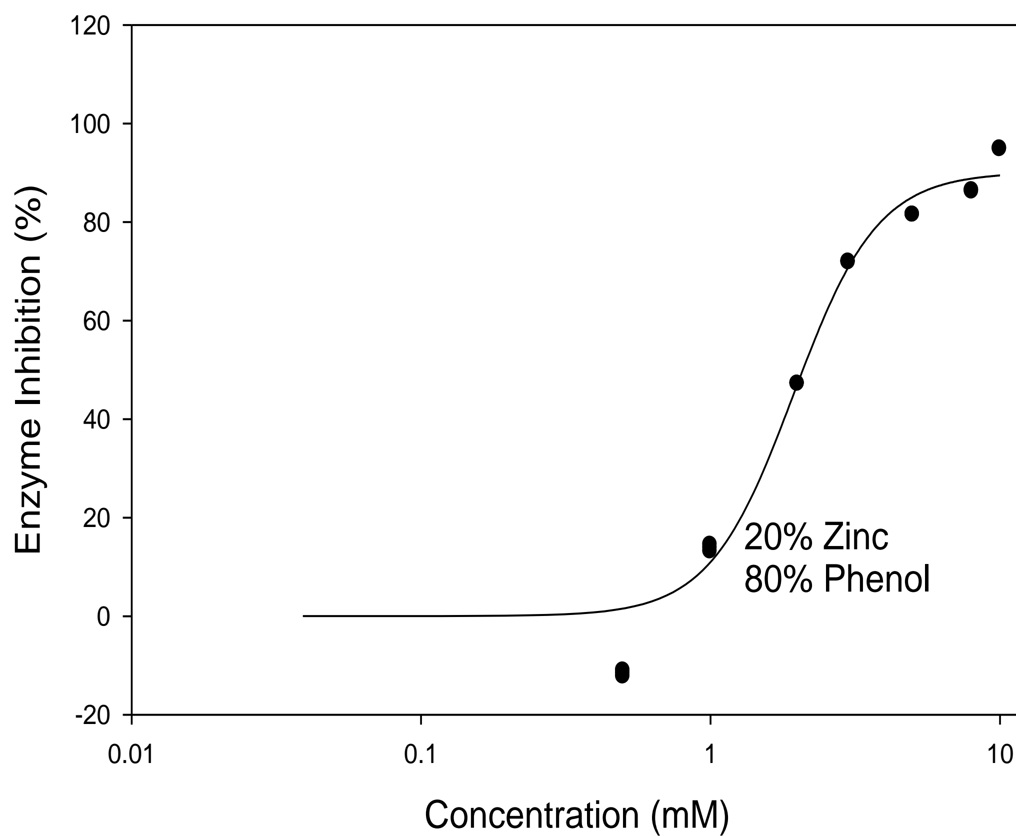


Figure 4.12: Toxicity of binary mixture of zinc and phenol on *Saprochaete* sp.

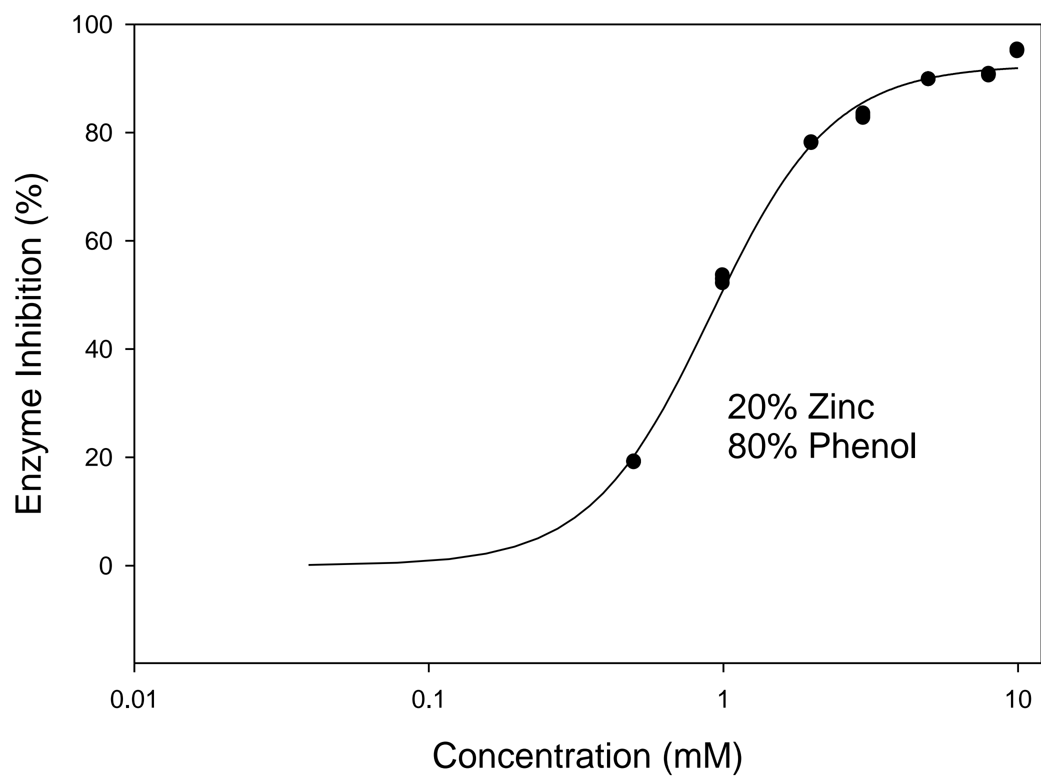


Figure 4.13: Toxicity of binary mixture of zinc and phenol on *Cryptococcus* sp.

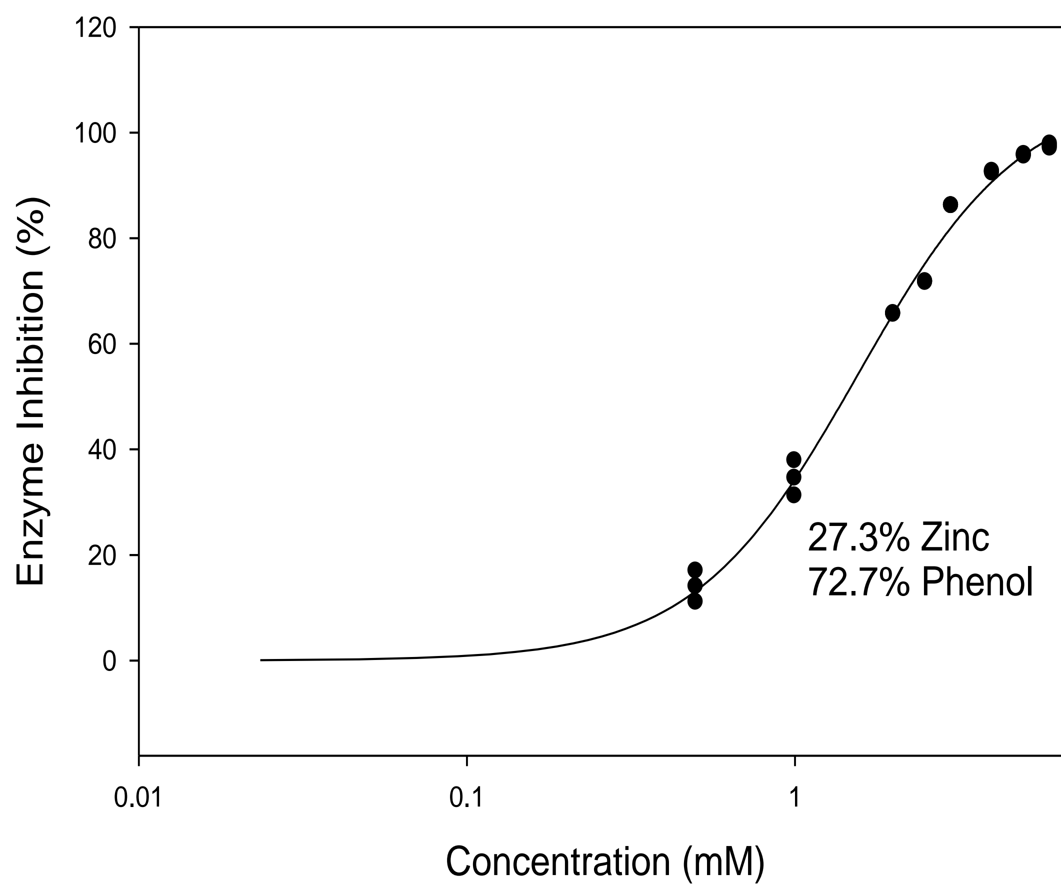


Figure 4.14: Toxicity of binary mixture of zinc and phenol on *Saprochaete* sp.

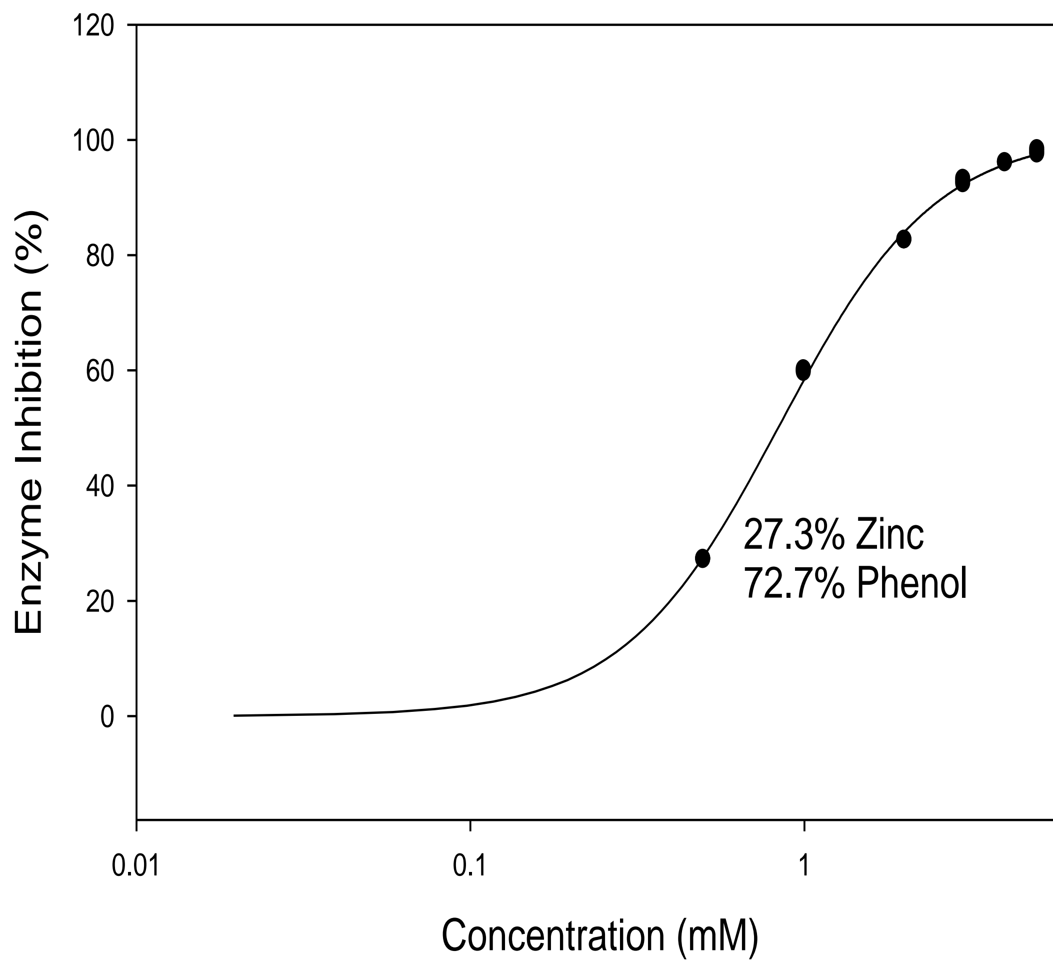


Figure 4.15: Toxicity of binary mixture of zinc and phenol on *Cryptococcus* sp.

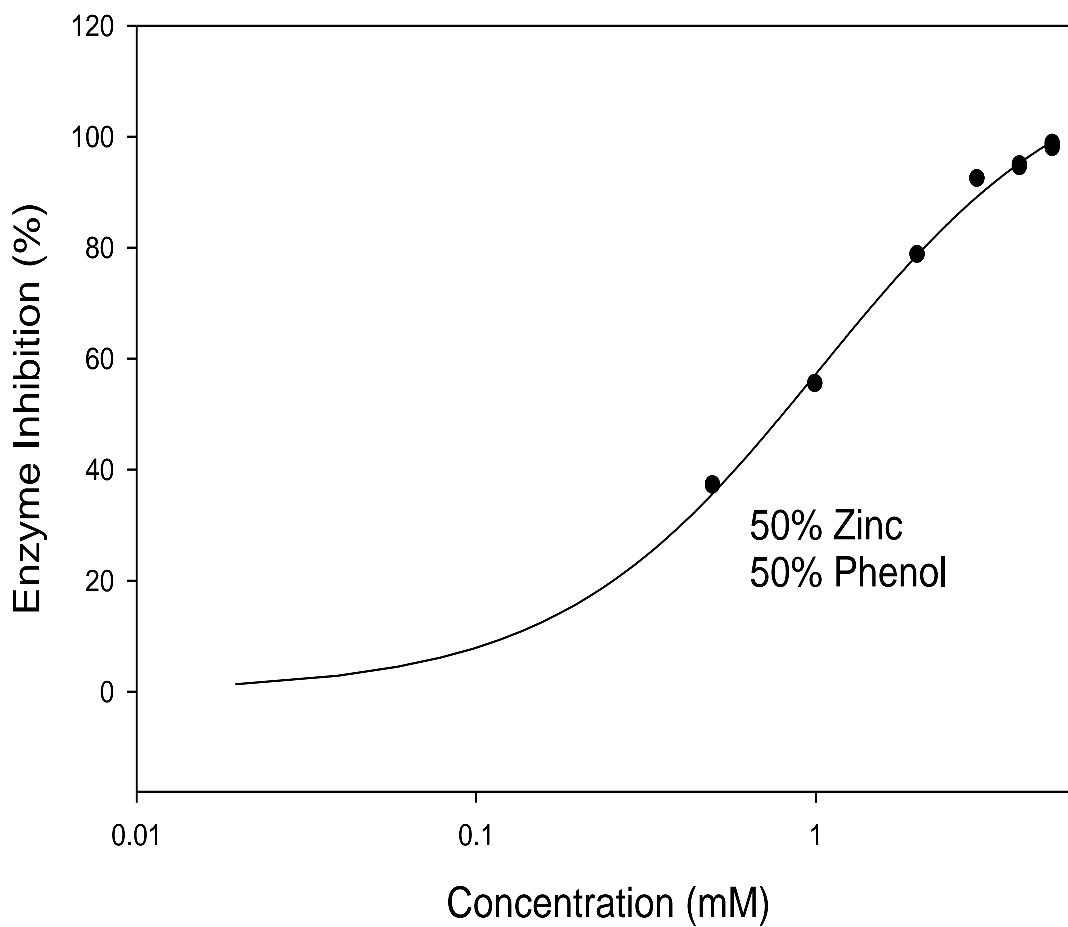


Figure 4.16: Toxicity of binary mixture of zinc and phenol on *Saprochaete* sp.

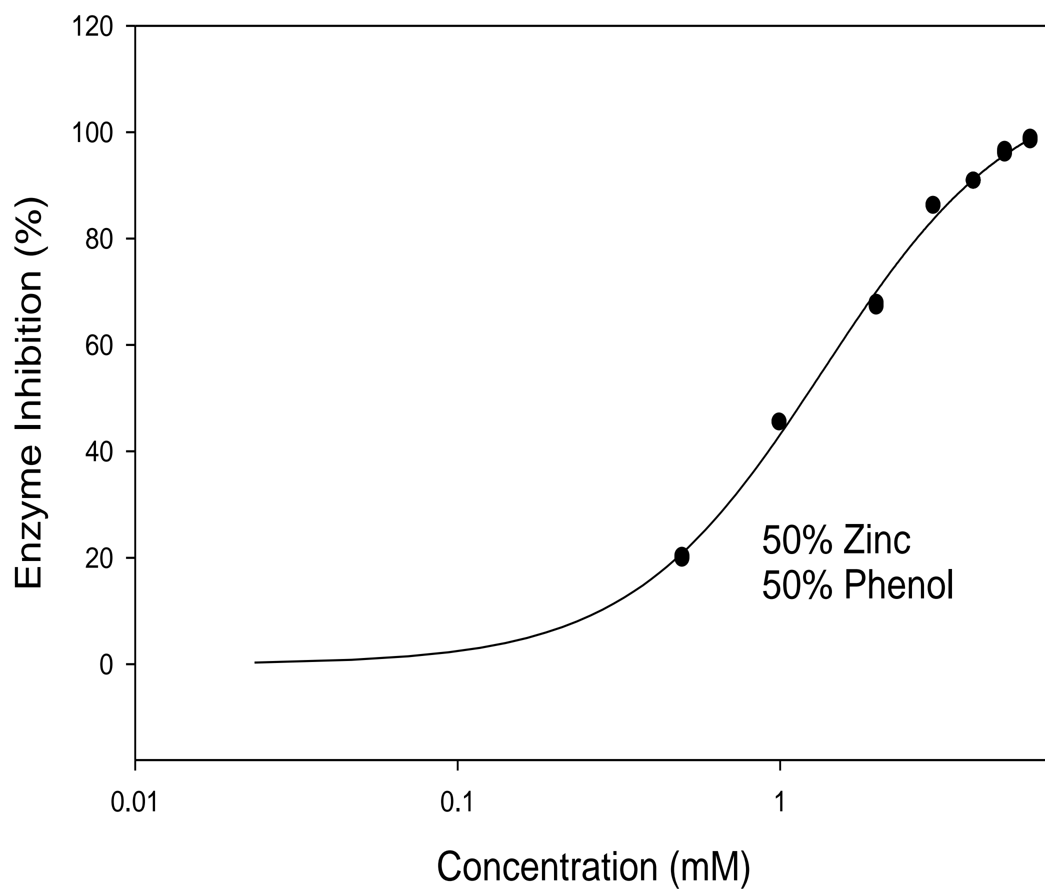


Figure 4.17: Toxicity of binary mixture of zinc and phenol on *Cryptococcus* sp.

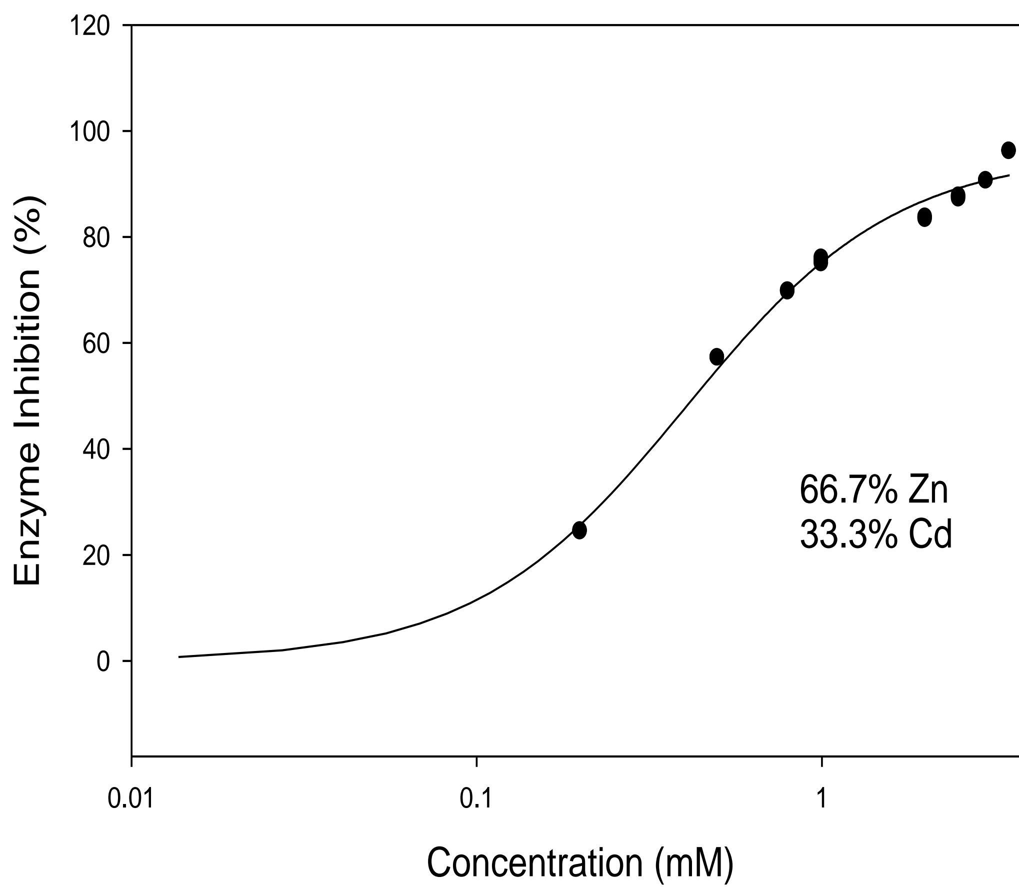


Figure 4.18: Toxicity of binary mixture of zinc and cadmium on *Cryptococcus* sp.

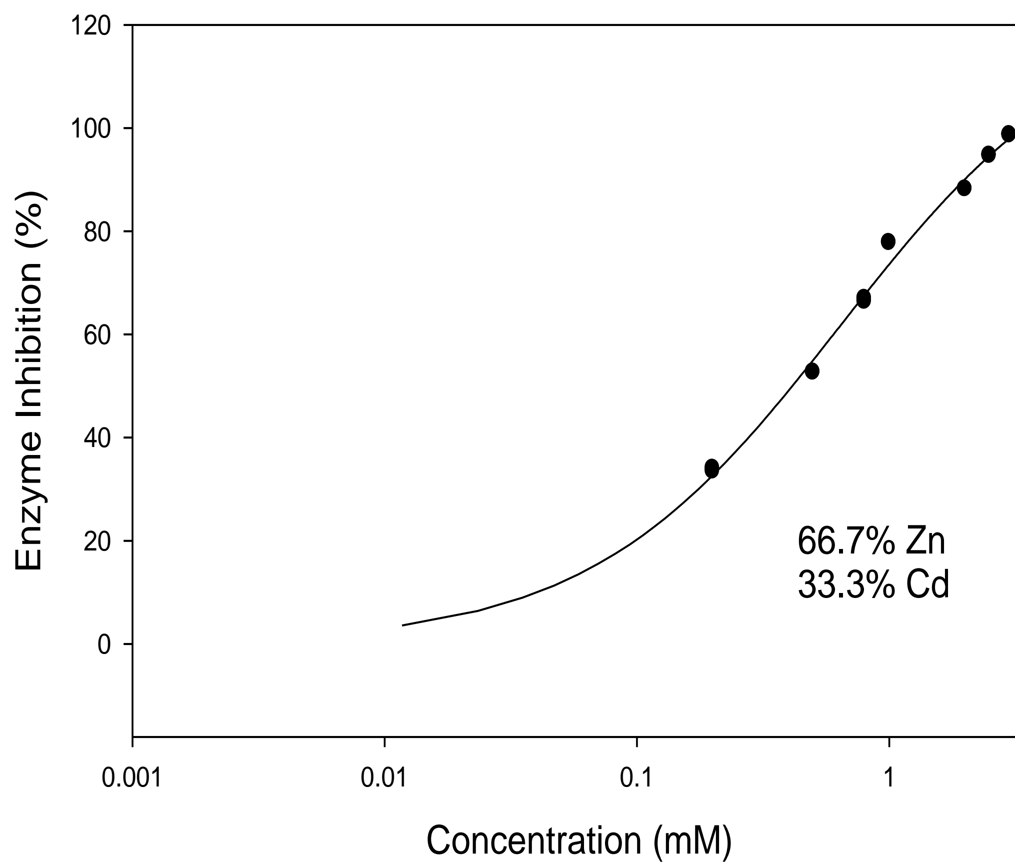


Figure 4.19: Toxicity of binary mixture of zinc and cadmium on *Saprochaete* sp**.**

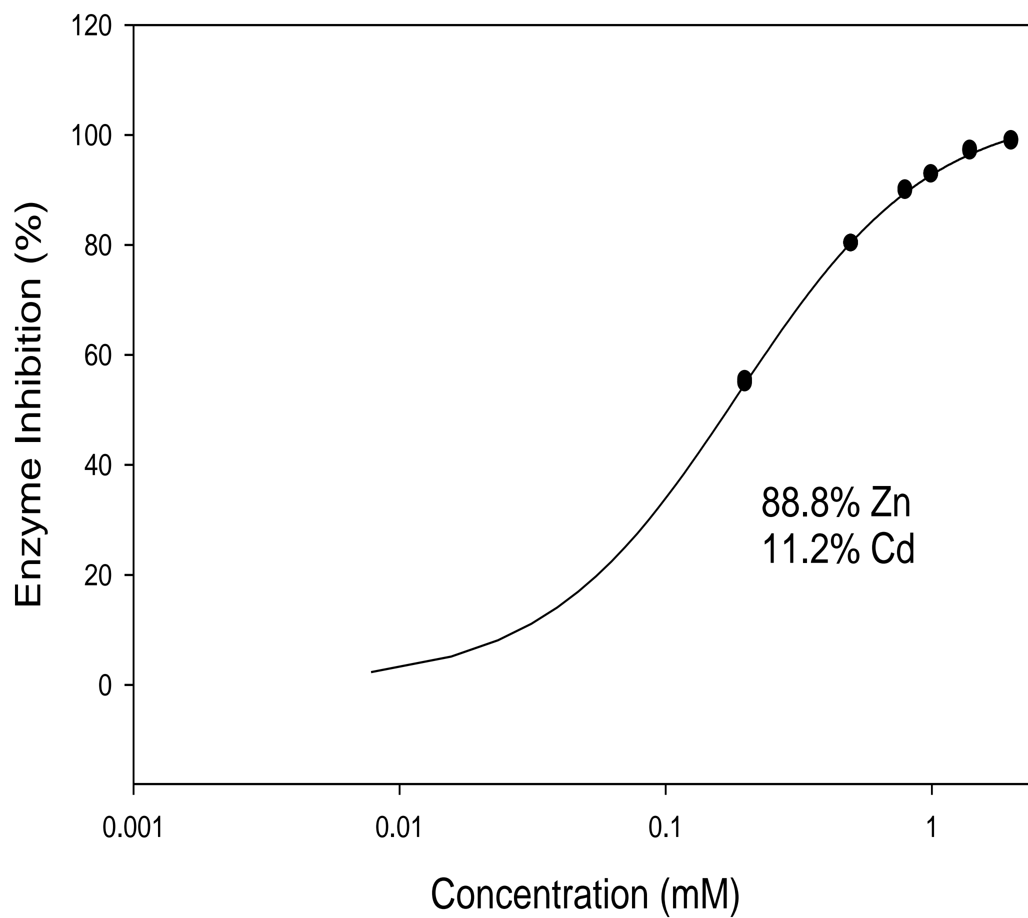


Figure 4.20 Toxicity of binary mixture of zinc and cadmium on *Saprochaete* sp.

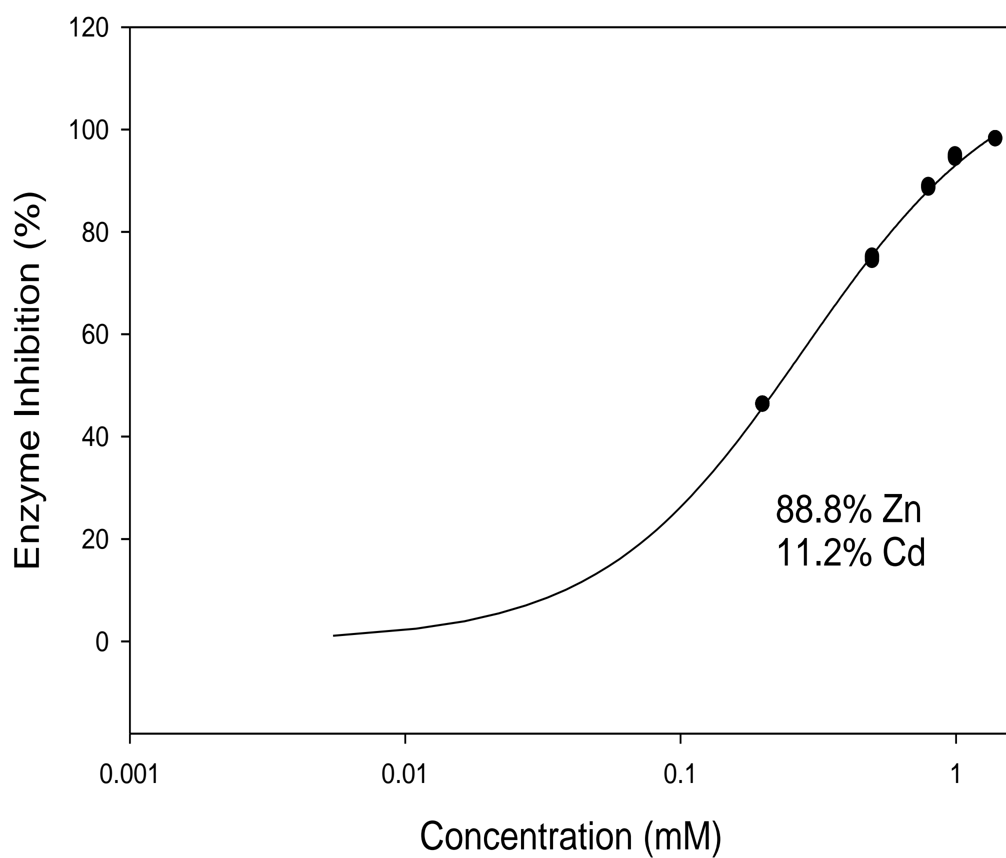


Figure 4.21: Toxicity of binary mixture of zinc and cadmium on *Cryptococcus* sp.

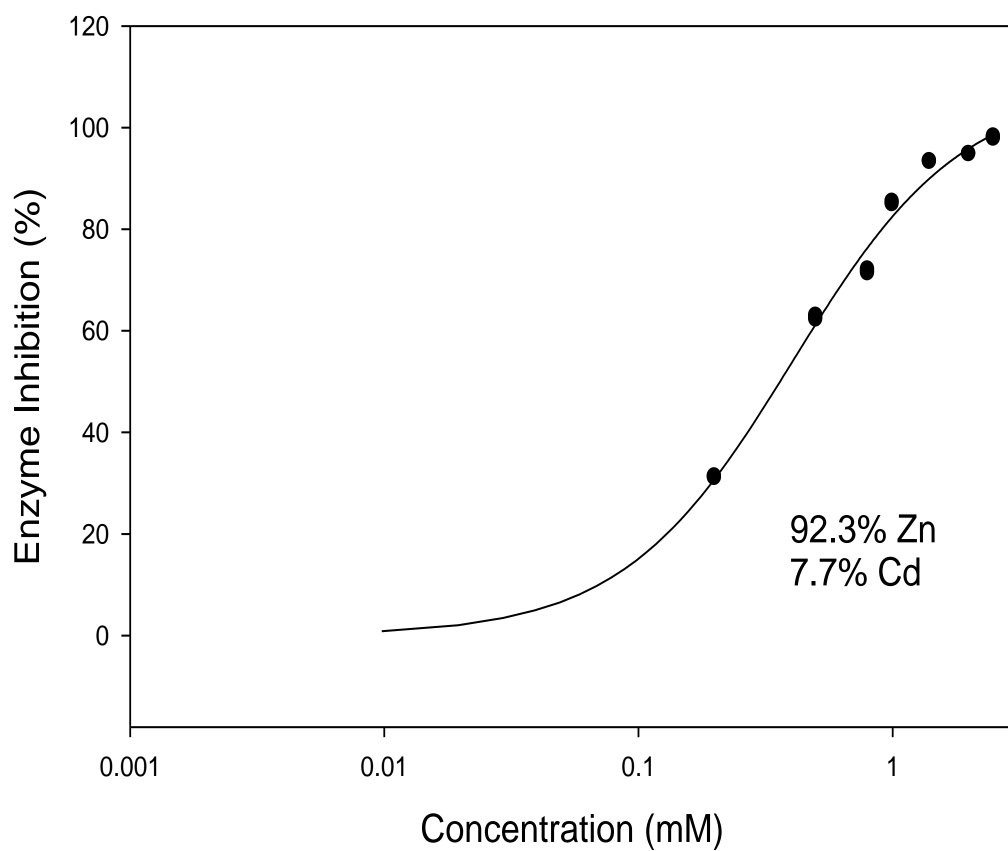


Figure 4.22: Toxicity of binary mixture of zinc and cadmium on *Saprochaete* sp.

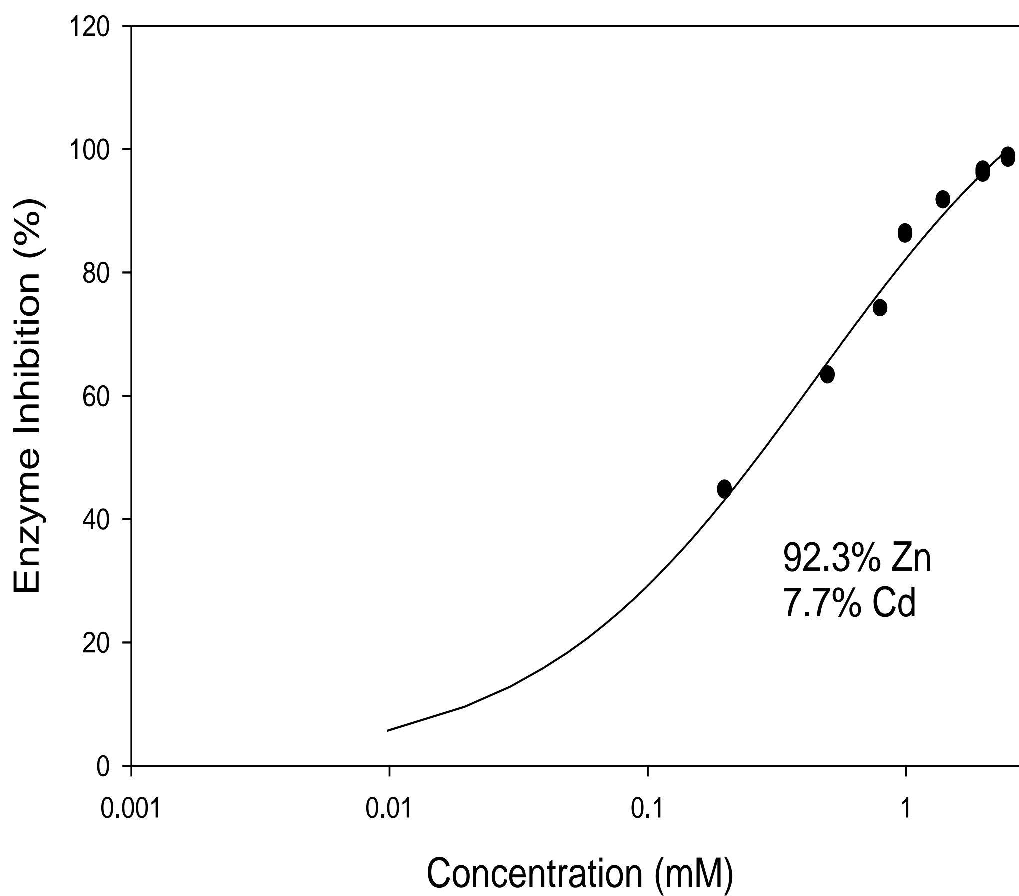


Figure 4.23: Toxicity of binary mixture of zinc and cadmium on *Cryptococcus* sp.

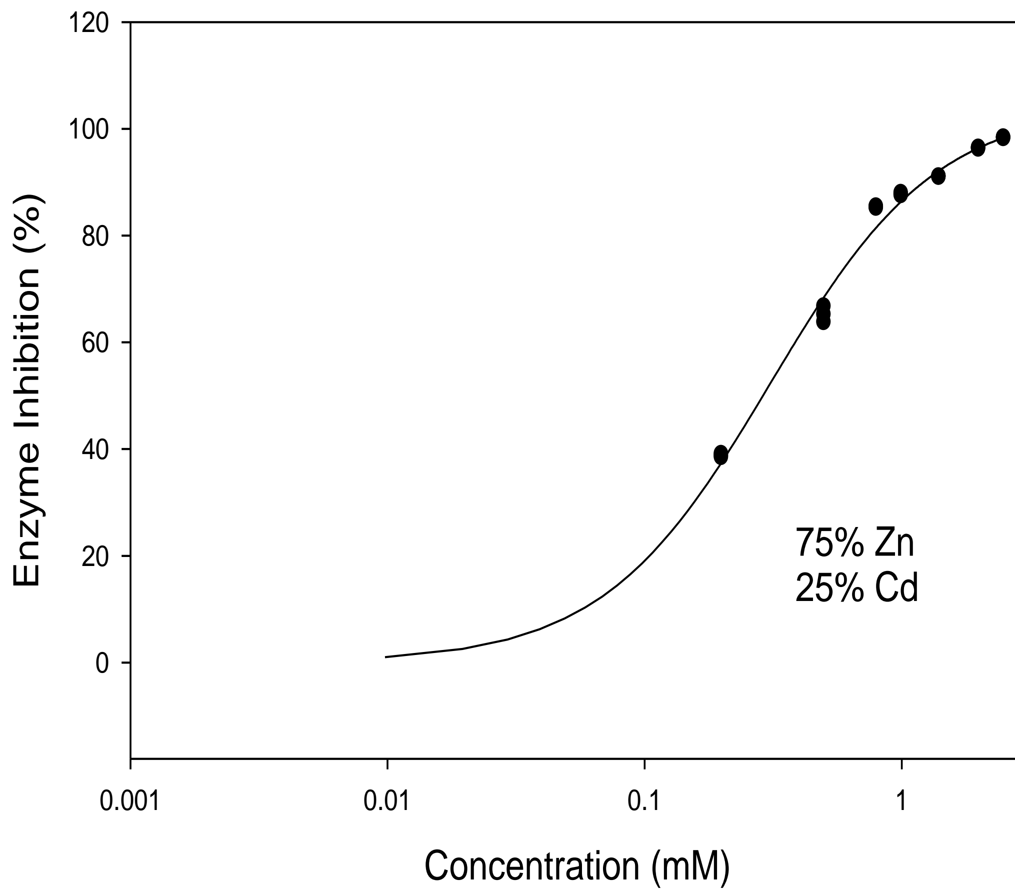


Figure 4.24: Toxicity of binary mixture of zinc and cadmium on *Saprochaete* sp.

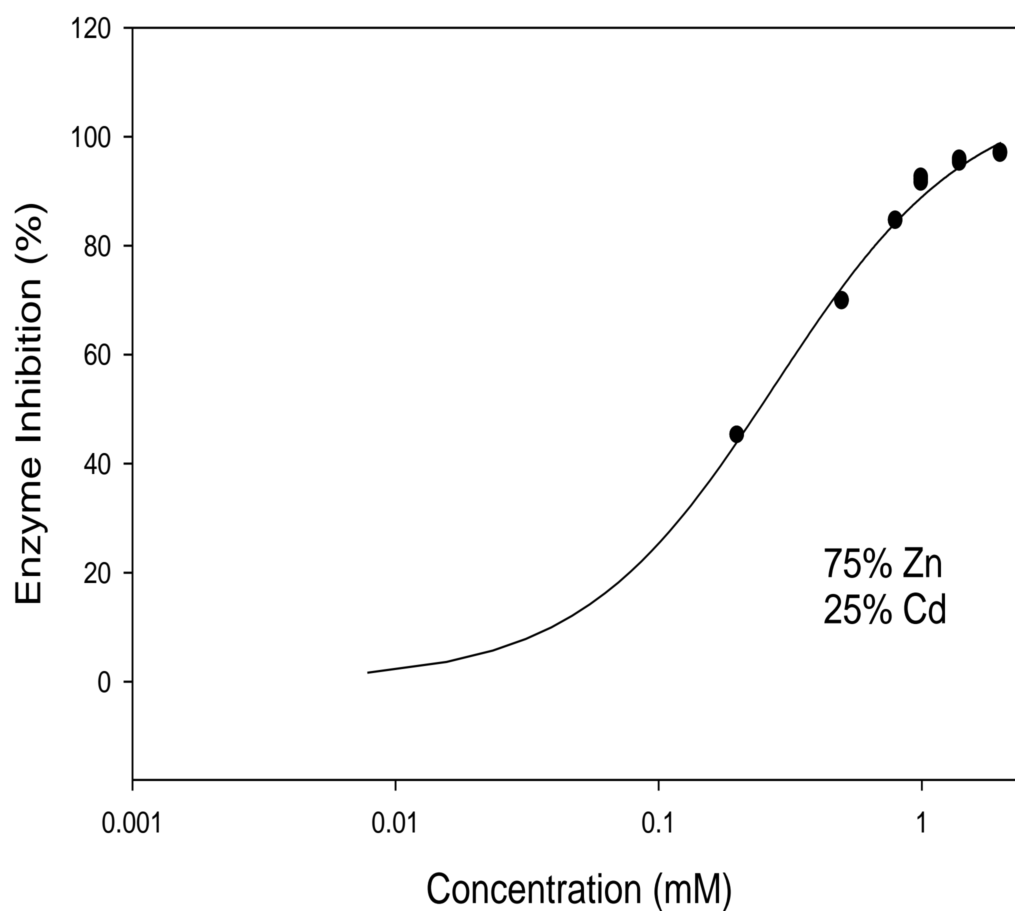


Figure 4.25 Toxicity of binary mixture of zinc and cadmium on *Cryptococcus* sp.

4.1.7 Toxicity of ternary mixtures of chemicals to the yeast strains

Figures 4.26, 4.27, 4.28, 4.29, 4.30, 4.31, 4.32 and 4.33 shows the model fit curve for the assessment of the toxic effects of ternary mixtures of zinc, cadmium and phenol on dehydrogenase activity of phenol-utilizing yeasts. The ternary mixtures of zinc, phenol and cadmium showed progressive inhibitory effects on the dehydrogenase (enzyme) activity of the test yeast as the concentration increases.

4.1.8 Equi-effect concentration

The Equi-effect (EE_{50}) mixtures of zinc and phenol, zinc and cadmium are shown in figure 4.34, 4.35, 4.36, 4.37, 4.38 and 4.39 for both yeasts. The result shows that there is an inhibitory effect on the dehydrogenase activity of the yeasts as the concentration of the mixtures increases.

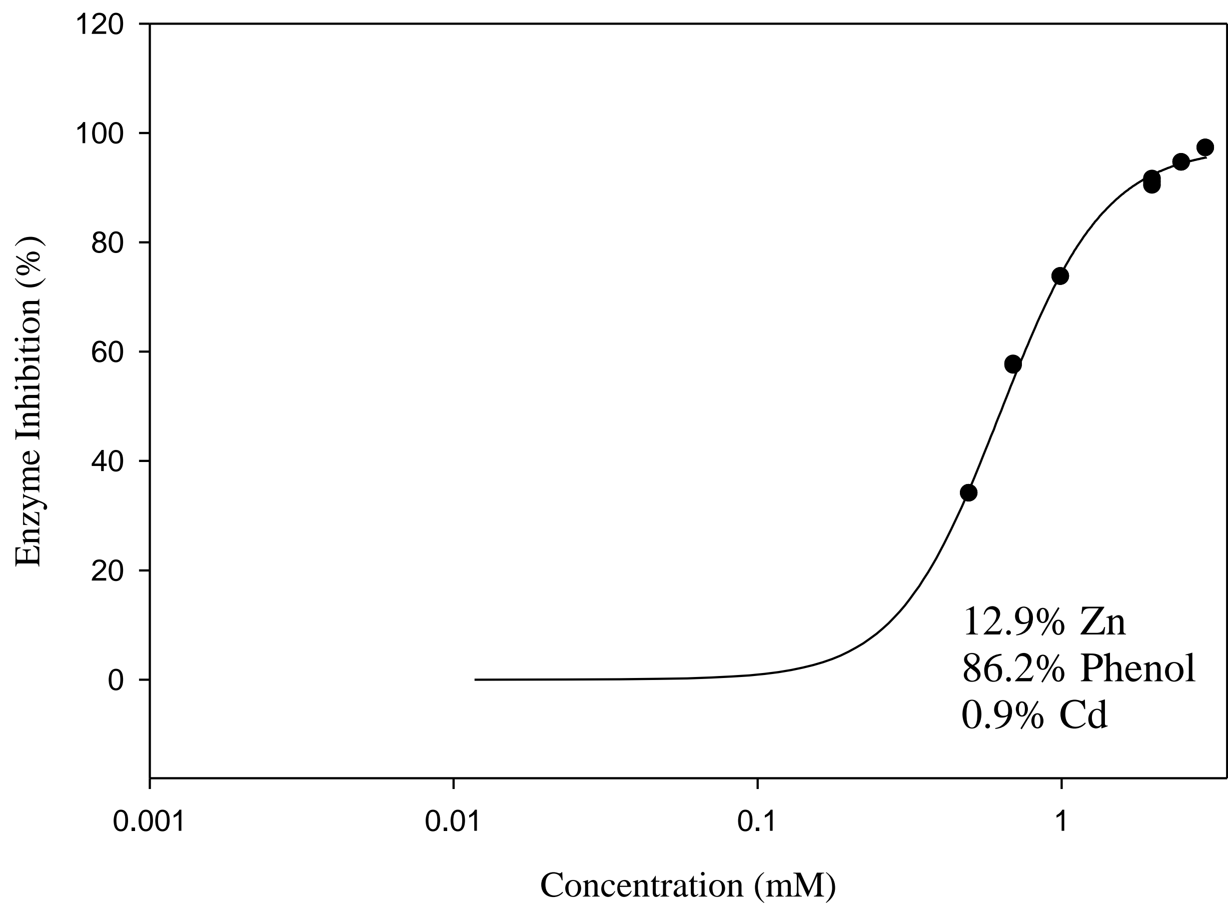


Figure 4.26: Toxicity of ternary mixtures of zinc, phenol and cadmium on *Saprochaete* sp

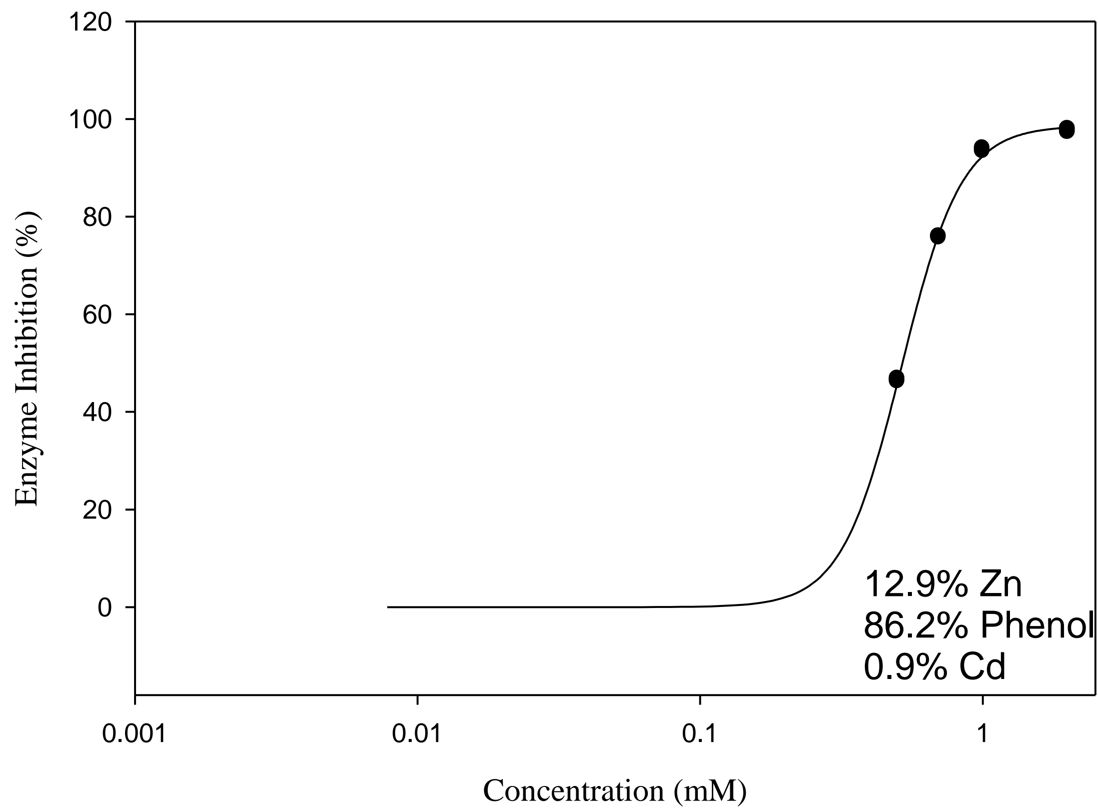


Figure 4.27: Toxicity of ternary mixtures of zinc, phenol and cadmium on *Cryptococcus* sp

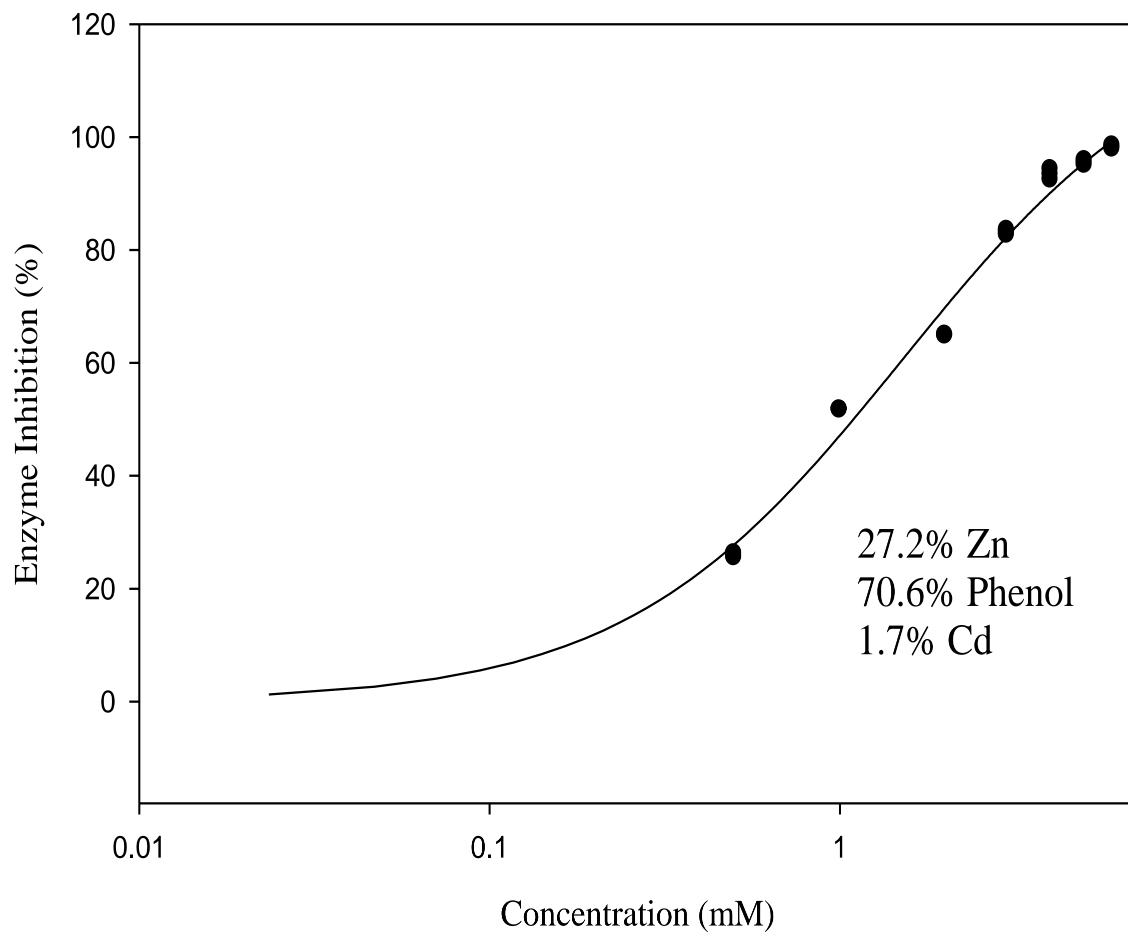


Figure 4.28: Toxicity of ternary mixtures of zinc, phenol and cadmium on *Saprochaete* sp.

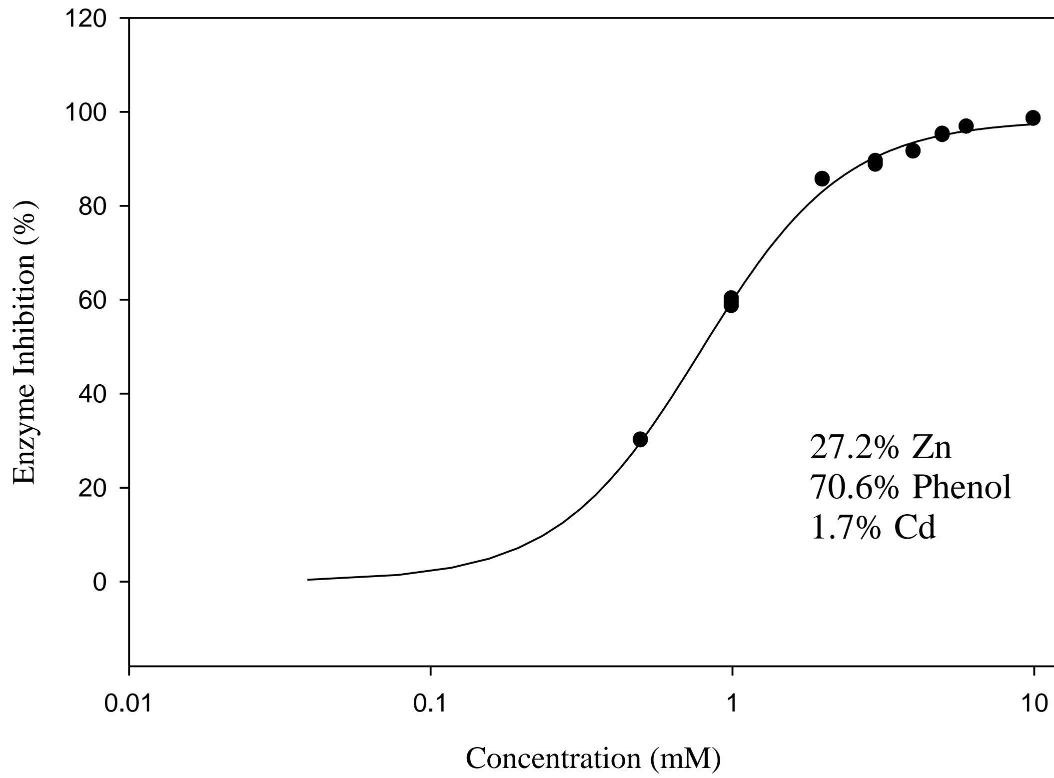


Figure 4.29: Toxicity of ternary mixtures zinc, phenol and cadmium on *Cryptococcus* sp

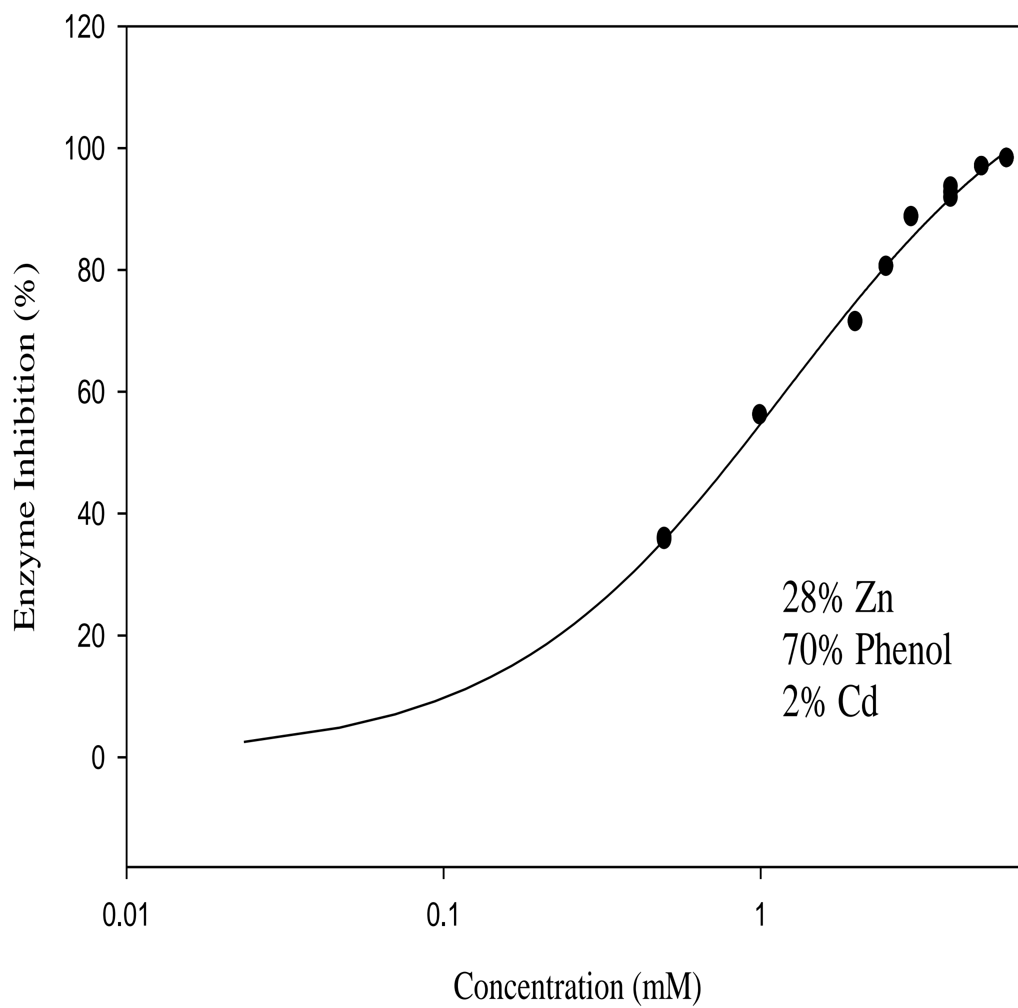


Figure 4.30: Toxicity of ternary mixtures of zinc, phenol and cadmium on *Saprochaete* sp.

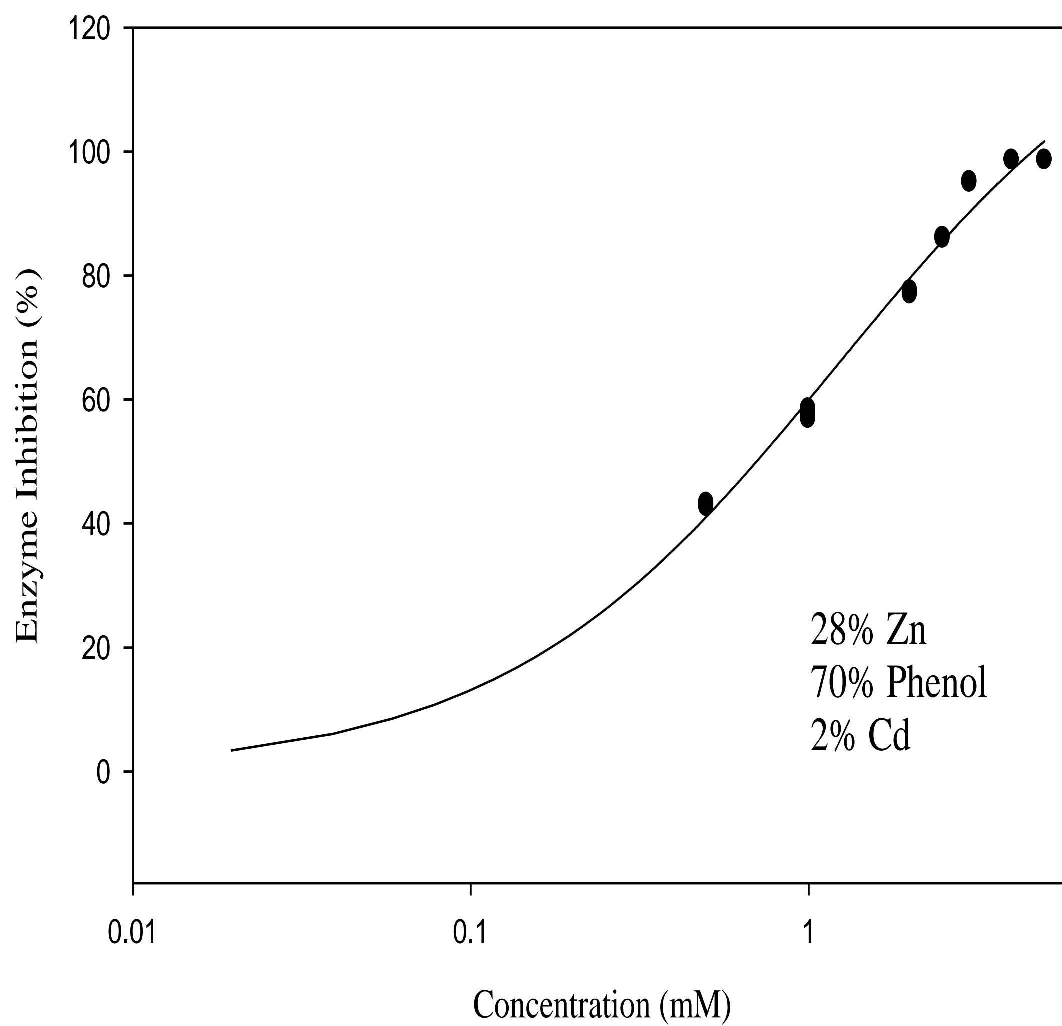


Figure 4.31: Toxicity of ternary mixtures of zinc, phenol and cadmium on *Cryptococcus* sp.

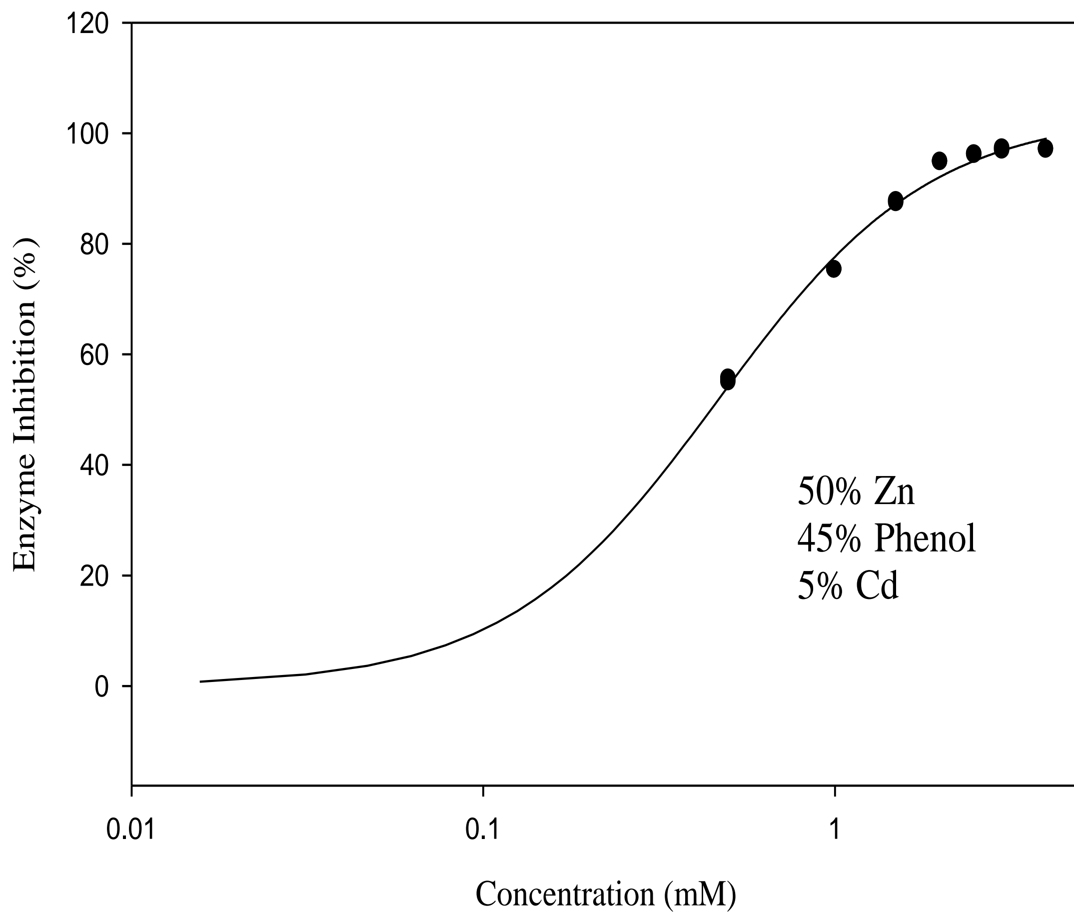


Figure 4.32: Toxicity of ternary mixtures of zinc, phenol and cadmium on *Saprochaete* sp.

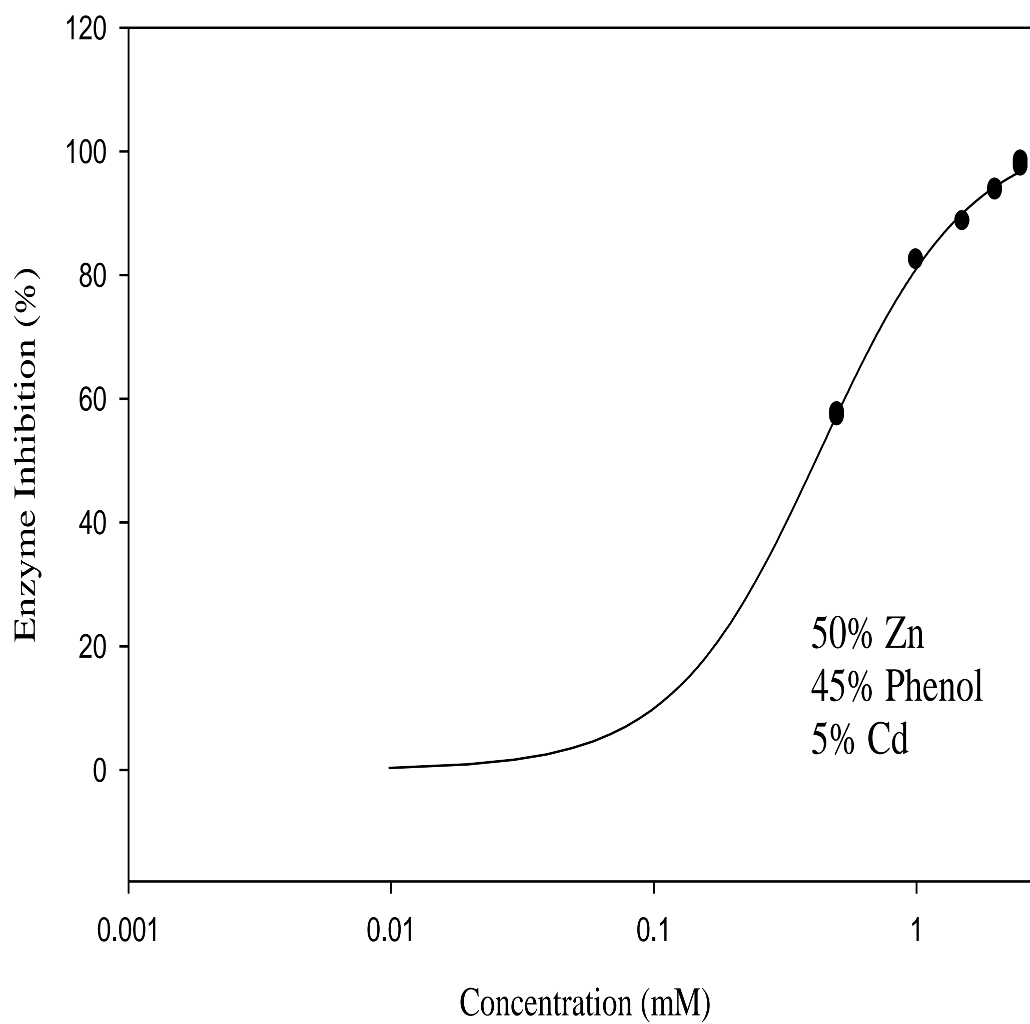


Figure 4.33: Toxicity of ternary mixtures of zinc, phenol and cadmium on *Cryptococcus* sp.

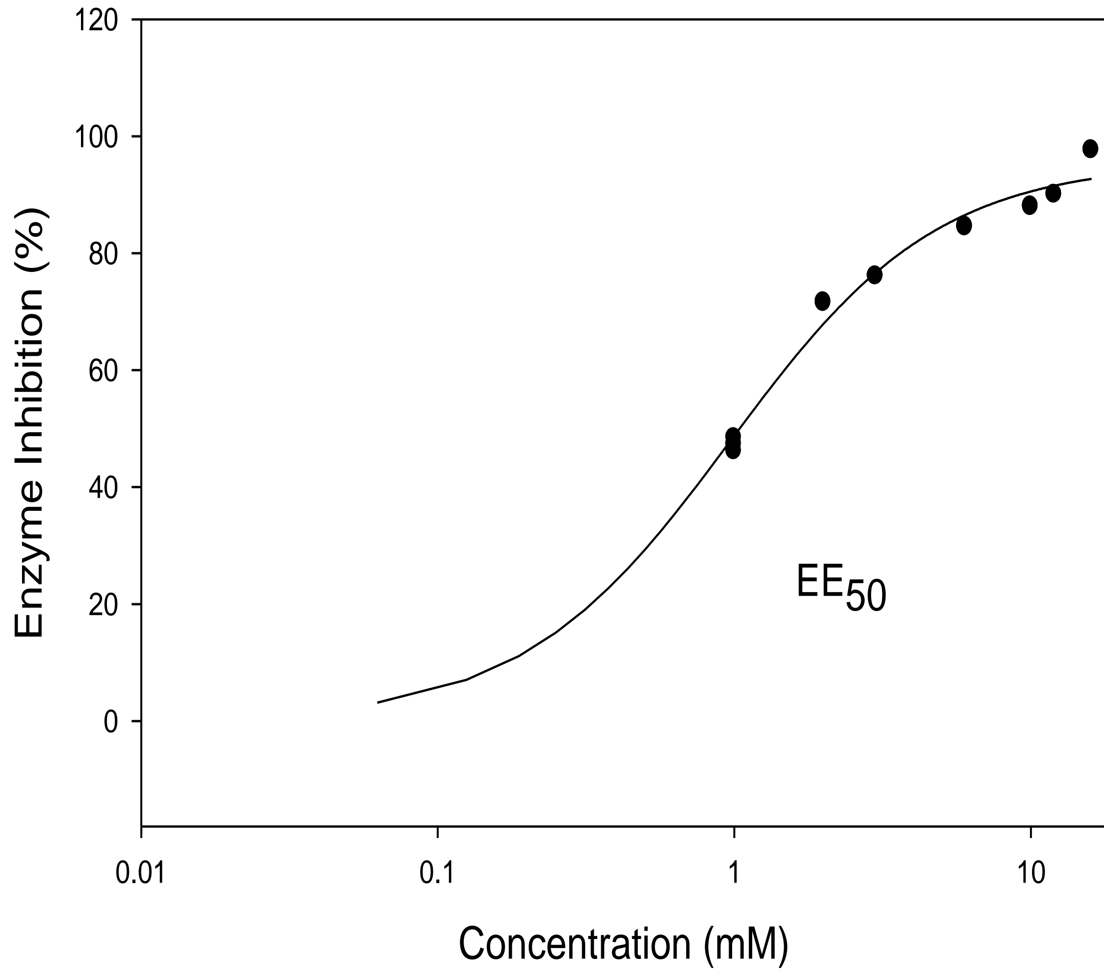


Figure 4.34: Equieffect (EE_{50}) mixture of zinc and phenol on *Saprochaete* sp.

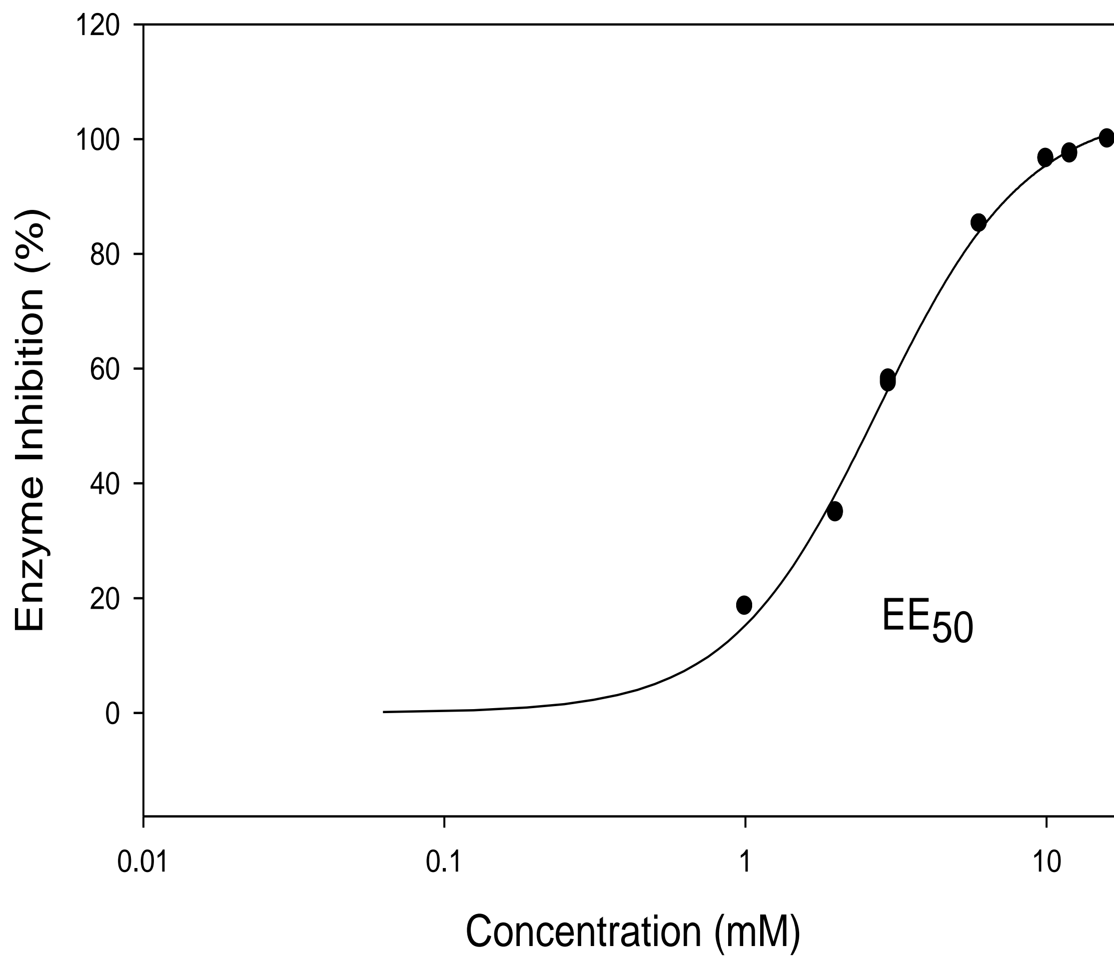


Figure 4.35: Equieffect (EE_{50}) mixture of zinc and phenol on *Cryptococcus* sp.

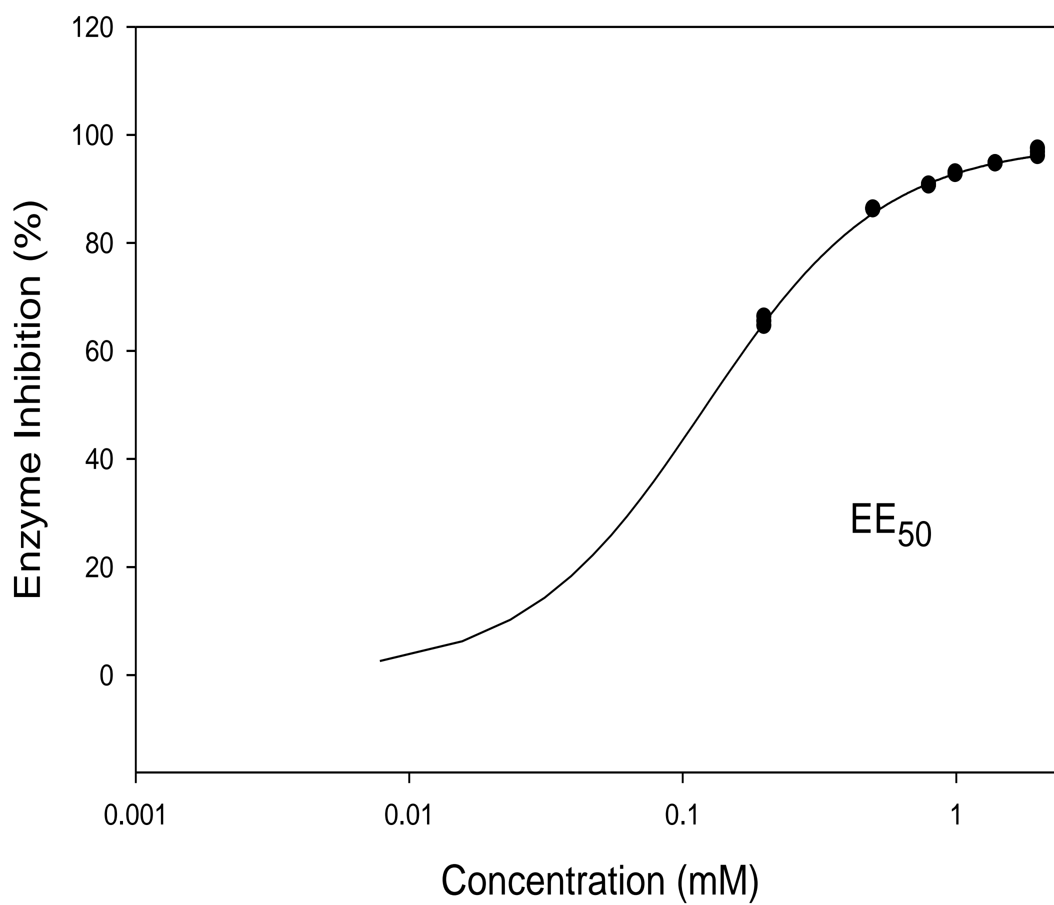


Figure 4.36: Equieffect (EE_{50}) mixture of zinc and cadmium on *Saprochaete* sp.

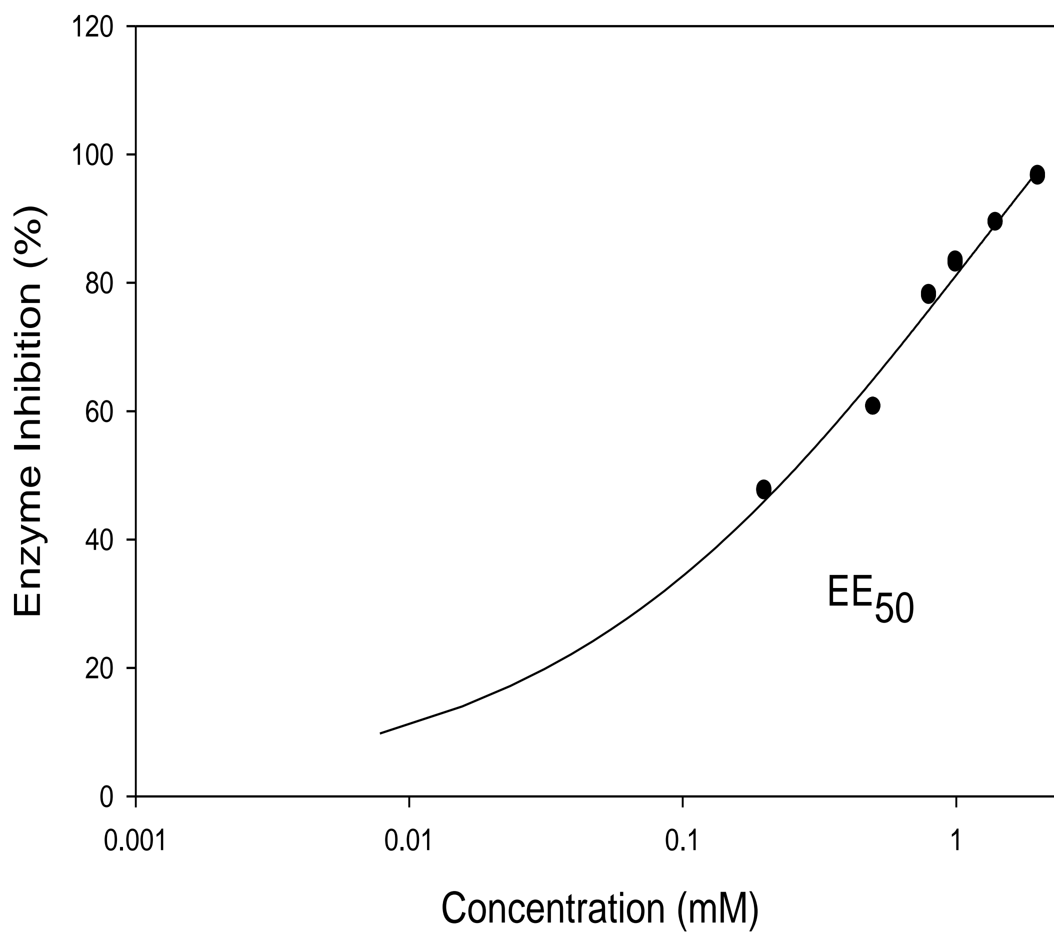


Figure 4.37: Equieffect (EE_{50}) mixture of zinc and cadmium on *Cryptococcus* sp.

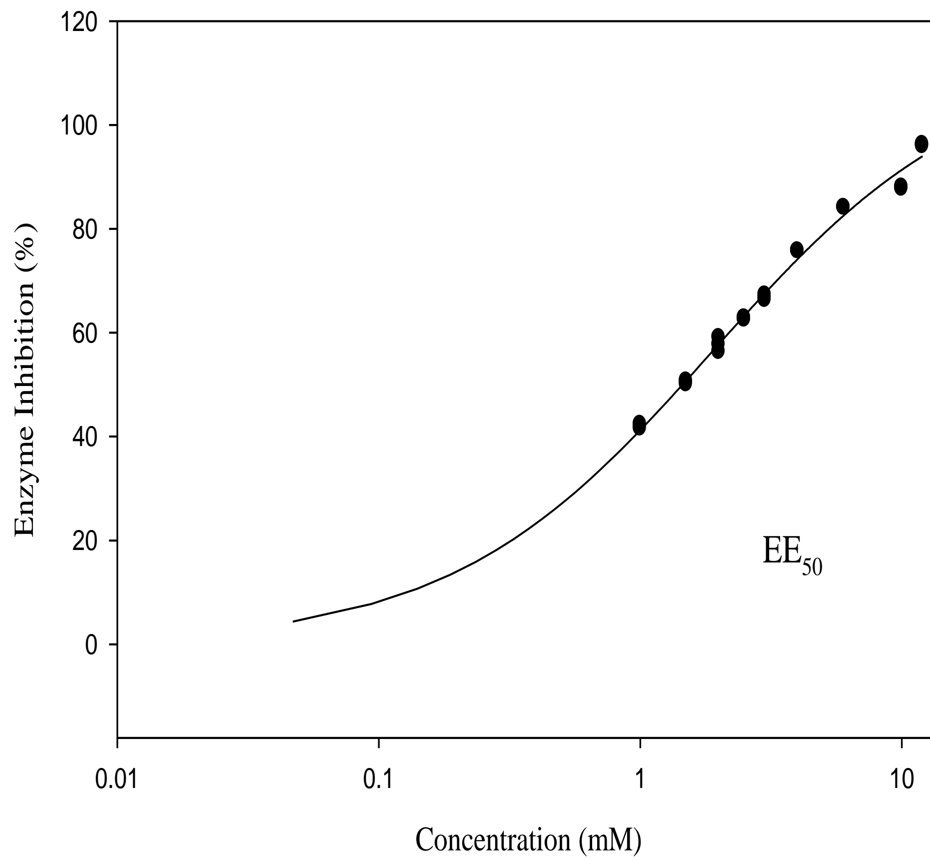


Figure 4.38: Equieffect (EE_{50}) mixture of zinc, phenol and cadmium on *Saprochaete* sp.

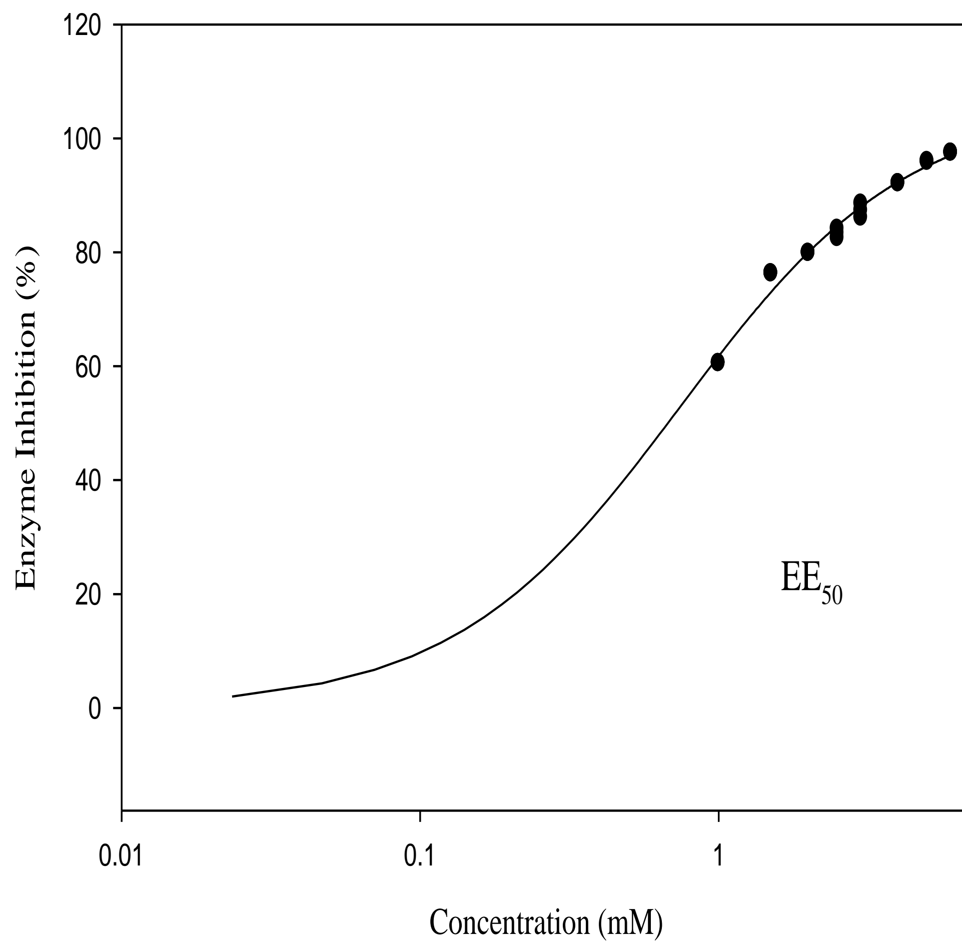


Figure 4.39: Equieffect (EE_{50}) mixture of zinc, phenol and cadmium on *Cryptococcus* sp.

4.1.9 Toxicity threshold (IC_{50}) of the chemicals and their combinations on enzyme activity of yeast strains

The toxicity threshold for the single, binary and ternary mixtures of the chemicals on the enzyme activity of both *Cryptococcus* sp. and *Saprochaete* sp., are shown in Table 4.5. For the single chemicals, cadmium has an IC_{50} value of 0.075 mM for *Saprochaete* sp. and 0.09 mM for *Cryptococcus* sp. This shows that cadmium had higher toxic effect on the yeasts than zinc and phenol with 0.385 mM, 0.358 mM and 6.110 mM, 6.489 mM respectively. For the binary mixtures, mixture ratio of 5.9% zinc + 94.1% phenol had lowest toxicity on the dehydrogenase activity of *Saprochaete* sp with IC_{50} of 2.255 mM and 1.505 mM for *Cryptococcus* sp, with the same mixture ratio. Mixture ratios of 88.8% zinc +11.2% cadmium gave the highest toxicity for *Saprochaete s*p with IC_{50} of 0.185 mM while mixtures ratios of 75% zinc + 25% cadmium recorded the highest toxicity for *Cryptococcus* sp with IC_{50} of 0.276 mM. For the ternary mixtures, mixture ratio of 50% zinc + 45% phenol +5% cadmium exerted the highest toxicity on both *Saprochaete* sp and *Cryptococcus* sp with IC_{50} of 0.466 mM and 0.43 mM respectively, while mixture ratio of 27.2% zinc + 71.1% phenol +1.7% cadmium had the lowest toxicity with IC_{50} of 1.151 mM for *Saprochaete* sp and IC_{50} of 1.215 mM on mixture ratio of 28% zinc + 70% phenol + 2% cadmium for *Cryptococcus* sp.

Toxicity of Equi-Effect (EE_{50}) mixtures was highest on dehydrogenase activity of *Saprochaete* sp with IC_{50} of 0.119 mM for binary mixtures of zinc and cadmium, while it exerted a low toxic effect on *Cryptococcus* sp with IC_{50} of 2.742 mM for mixtures of zinc and phenol. The statistical analysis in appendix iii showed that there is no significant difference (0.0803) in the IC_{50} of both organisms at 0.5% significant level.

Table 4.6: Toxicity thresholds (IC_{50}) of chemicals as single, binary, ternary and equi-effects mixtures on dehydrogenase activity of the test yeasts.

Chemical/ Ratio	IC_{50}(mM)	
	Saprochaete sp.	*Cryptococcus* sp.
Zinc	0.385 ± 0.012	0.358±0.025
Phenol	6.11±0.217	6.489±0.387
Cadmium	0.075±0.012	0.09±0.005
66.6% zinc + 33.3% cadmium	0.638±0.113	0.404±0.031
88.8% zinc + 11.2% cadmium	0.185±0.022	0.280±0.007
92.3% zinc + 7.7% cadmium	0.403±0.024	0.445±0.124
75% zinc + 25% cadmium	0.307±0.0132	0.276±0.052
5.9% zinc + 94.1% phenol	2.255±0.064	1.505±0.077
20% zinc + 80% phenol	1.937±0.083	0.906±0.051
27.3% zinc + 72.7% phenol	1.554±0.064	0.844±0.042
50% zinc + 50% phenol	1.022±0.085	1.355±0.195
12.9% zinc +86.2% phenol + 0.9% cadmium	0.627±0.014	0.50±0.0186
27.2% zinc + 71.1% phenol + 1.7% cadmium	1.151±0.289	0.786±0.044
28% zinc + 70% phenol + 2% cadmium	1.12±0.119	1.215±0.370
50% zinc + 45% phenol + 5% admium	0.466±0.016	0.43±0.057
EE_{50} zinc + cadmium + phenol	1.89±0.362	0.746±0.074
EE_{50} zinc + cadmium	0.119±0.016	1.120±1.96
EE_{50} Zinc + Phenol	0.966±0.080	2.747±0.176

4.1.10 Toxic interaction of the combinations of the chemicals on the yeast strains dehydrogenase activity

Toxic interactions of the binary, ternary and equi-effect mixtures of the chemicals on dehydrogenase activity of the yeasts are shown in Table 4.6. For binary mixtures of zinc and phenol, all the mixtures ratios evaluated showed antagonistic interaction eexcept 5.9% zinc + 94.1% phenol which showed synergistic effect for *Saprochaete* sp, while for *Cryptococcus* sp, all the mixtures ratios showed synergistic interaction with the exception of 50% zinc + 50% phenol, which show antagonistic interaction. For the binary mixtures of zinc and cadmium, all the mixtures ratios showed antagonistic interaction with the exception of 88.8% zinc + 11.2% cadmium, which exhibited synergistic effect for *Saprochaete* sp, while for *Cryptococcus sp*, all the mixtures ratios exhibited antagonistic interaction. For the ternary mixtures, all the mixtures ratios showed synergistic interaction for both *Saprochaete* sp and *Cryptococcus* sp.

Equi-effect concentration (EE_{50}) of binary mixtures of zinc and phenol exhibited synergistic effect on *Saprochaete* sp, while on *Cryptococcus* sp, marginally additive effect was observed. For the equi-effect concentration (EE_{50}) of binary mixtures of zinc and cadmium, synergistic effect was observed in both *Saprochaete* sp and *Cryptococcus* sp.

Table 4.6: Toxic Interactions of the binary, ternary and equi- effect mixtures of the test chemicals on the yeasts.

Mixture/ Ratio	*Saprochaete* sp.		*Cryptococcus* sp.	
	Toxic Index (TI)	Interaction	Toxic Index (TI)	Interaction
66.6% zinc + 33.3% cadmium	3.938	Antagonistic	2.248	Antagonistic
88.8% zinc + 11.2% cadmium	0.703	Synergistic	1.043	Antagonistic
92.3% zinc + 7.7% cadmium	1.380	Antagonistic	1.528	Antagonistic
75% zinc + 25% cadmium	1.621	Antagonistic	1.345	Antagonistic
5.9% zinc + 94.1% phenol	0.693	Synergistic	0.466	Synergistic
20% zinc + 80% phenol	1.343	Antagonistic	0.618	Synergistic
27.3% zinc + 72.7% phenol	1.512	Antagonistic	0.731	Synergistic
50% zinc + 50% phenol	1.411	Antagonistic	1.997	Antagonistic
12.9% zinc + 86.2% phenol + 0.9% cadmium	0.369	Synergistic	0.139	Synergistic
27.2% zinc + 71.1% phenol + 1.7% cadmium	0.607	Synergistic	0.147	Synergistic
28% zinc + 70% phenol + 2% cadmium	0.909	Synergistic	0.834	Synergistic
EE_{50} zinc + cadmium	0.517	Synergistic	5.00	Synergistic
EE_{50} zinc + phenol	0.297	Synergistic	0.802	Marginally Additive

4.2 DISCUSSION

The isolates from the hydrocarbon impacted soil collected from Orji Mechanic Village in Owerri, Imo State of Nigeria included *Saprochaete* sp and *Cryptococcus* sp which were identified on the basis of morphological, microscopic and biochemical characteristics (Campbell *et al*., 2013).

Phenol degrading yeasts and mould that has been used in previous studies include *Candida tropicalis, Pichia guilliermondii, Meyerozyma guilliermondii* (Karimi, and Hassanshahian, 2016), and *Aspergillus niger* (Neha and Vikas, 2012).

In this study, growth of *Saprochaete* sp and *Cryptococcus* sp on different concentrations of phenol (0.1, 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0mM) amended mineral salt agar, shows that increased phenol concentration decreased the yeast growth and the lowest growth obtained in both isolates are $3.3 x 10^3$and $2.3 x 10^3$ cfu/ml at phenol concentration of 0.5 mM. Abd El-Zaher *et al*. (2011), reported the effect of different concentrations of phenol on growth of some fungi isolated from contaminated soil, which evaluated that increase in phenol concentration decreases fungal growth.

In this study, it was observed that the isolates *Saprochaete* sp. and *Cryptococcus* sp. utilized maximum amount of certain hydrocarbons. The isolated organisms utilized maximum amount of crude oil and diesel which was evidence in their survival in the sampled soils and in vitro investigations. Sandhu *et al.* (2016) reported the hydrocarbon degrading potentiality of indigenous fungal isolates which indicated that showed high potency to degrade petroleum hydrocarbon. Damisa *et al*. (2013) also reported that, *Aspergillus niger* and *Candida* sp. were found to degrade crude oil more efficiently, in their work biodegradation of Petroleum by fungi isolated from unpolluted tropical soil.

.The type of degradation responses obtained from the performance of the organisms was found to be broadly dependent upon the concentration of phenol in mineral salt broth (MSB). Increase in the concentration of the phenol inhibits the growth of the organisms which leads to increase in the time of degradation. The inhibition of microbial growth and biodegradation of phenol is a well-known phenomenon and has been reported in many organisms (Oboirien *et al*., 2005; Okpokwasili and Nweke, 2006; Agarry *et al*., 2010). The effect of

different phenol concentrations on the growth of yeast strains showed that when the concentration of phenol was increased, the rate of degradation was decreased. Varma and Gaikwad (2009)) in their work investigated the degradation of phenol (200 mg L^{-1}) by *Candida tropicalis* strain NCIM 3556, showing that this strain, in16 h, reached the maximum degradation (96.28%) and after 48 h, the degradation was almost completed (99.88%). Liu *et al*. (2011) concluded that the yeast *T. montevideense* strain PHE1 well tolerated the toxicity of phenol concentrations above 2500 mg L^{-1}. *Saprochaete* sp degraded the phenol within fourteen days [14 d (336 h)] of incubation, while *Cryptococcus* sp degraded the phenol within eleven days [11 d (264 h)] of incubation, time-dependent degradation of organic compounds has been reported to be linked with concentration of the organic compound as observed by many authors (Colwell and Walker, 1977; Kotresha and Vidyasagar, 2008). This may be due to changes in the transport mechanism of the substrate across the cell membrane in response to high phenol concentration hence, diminished capacity to catabolize phenol (Nwanyanwu and Abu, 2013). There was variation in degradation of the phenol by *Saprochaete* sp and *Cryptococcus* sp., which may be due to differences in cell wall components or poor induction of phenol hydroxylase systems, since phenol hydroxylase is known to be the major site for phenol inhibition (Leonard and Lindley. 1999).

The values of specific growth rate, phenol degradation rate and specific degradation rate of the *Saprochaete* sp. and *Cryptococcus* sp. showed decrease in the specific growth rate as the initial concentrations of the phenol increases. Santos and Linardi (2001) in their work on phenol degradation of yeast isolated from industrial effluent, reported that *Trichosporon* LE3 grew in the presence of up to 20 mM phenol, but showed a reduction in specific growth rate at concentrations higher than 11mM, suggesting an inhibitory effect of phenol at these concentrations. According to Dey and Mukherjee (2010), the higher the concentration of the growth-limiting substrate, the higher will be specific growth rate and the closer it will approach a maximum value. However, if this substrate shows inhibitory action, the continuous increment in specific growth rate should not happen, but rather a reduction in specific growth rate would occur, increasing substrate concentration (Dey and Mukherjee, 2010). The effect of different phenol concentrations on the growth of

yeasts showed that when the concentration of phenol was increased, the rate of degradation was decreased. Karimi and Hassanshahian (2016) reported that there is decrease in the rate of degradation with increase in initial phenol concentration. The degradation rate and specific degradation rate showed maximum phenol degradation at 500 mg/l by the isolates. This could be due to the fact that the phenol degrading enzymes activity is optimum at this concentration (Supriya1 and Neehar, 2014). Supriya1 and Neehar (2014), reported maximum phenolic degradation at 300 mg/l by *Aspergillum niger*.

Phenols are membrane damaging biocides (Okolo *et al*., 2007), causing loss of cytoplasmic membrane integrity and thus, disruption of membrane functions. Since dehydrogenases are membrane associated, loss of membrane integrity will ultimately affect their functions. The toxicity of the individual chemicals phenol, zinc and cadmium on dehydrogenase activity of both *Saprochaete* sp. and *Cryptococcus* sp. indicates progressive inhibition of the dehydrogenase activity in both yeasts as the concentrations of the chemicals increases. Cadmium exhibited sharp inhibitory effect on the dehydrogenase (enzyme) activities of the yeasts. This is in line with the report of Nweke and Okpokwasili (2010), where increase in phenol concentration decrease the dehydrogenase activity in the microbial community in refinery wastewater bacteria.

The results from the binary mixtures of zinc, phenol and zinc, cadmium and the ternary mixtures of the three chemicals at different ratios is in agreement with previous study carried out by Nweke and Okpokwasili (2014) reported that low dose stimulation of glyphosate, 4-Chlorophenol and 2, 4 dichlorophenol on dehydrogenase activity of *Rhizobium* species and Nweke *et al.* (2016), where glyphosate, phenol, 4-CP and 2, 4-DCP progressively inhibited dehydrogenase activity. In a study on the removal of phenol and zinc by *Candida* isolated from wastewater for integrated biological treatment, Mahgoub *et al*. (2015) reported low dose stimulatory effect of zinc and phenol on *Candida* sp.

Certain metals such as copper, cobalt, nickel, iron and zinc are essential for normal growth and metabolism. Zinc, copper, chromium and iron may influence the stability of the double helix of DNA, while forming hydrogen bonds (Choudhury and Srivastava, 2001). However, in excess of physiologically required levels, these metals become toxic and disrupt the biochemical activities of cells. The toxicity of zinc ion observed in

this study thus, corroborated reported toxic effects of metal ions (Choudhury and Srivastava, 2001). Zinc is known to be a potent inhibitor of the respiratory electron transport systems bacteria and eukaryotic organisms (Choudhury and Srivastava, 2001). In microbial community of *Vigna unguiculata,* IC_{50} of 0.256 ± 0.019 mM and 0.825 ± 0.118 mM was reported for Cd^{2+} and Fe^{2+} respectively (Nweke *et al*., 2007).

The inhibition of dehydrogenase activity of *Saprochaete* sp. and *Cryptococcus* sp. to zinc ion is in line with toxic effect of metals on prokaryotic and eukaryotic cells. Although the mechanism of this inhibition was not investigated, metal ions are known to inhibit the metabolic activities of yeasts (Nweke and Okpokwasili, 2010). Toxic effects includes the blocking of functional groups, displacement and substitution of essential metal ions for biomolecules, conformational modification, denaturation and inactivation of enzymes and distruptions of cellular and organellar membrane integrity Nweke and Okpokwasili (2010). The response of *Saprochaete* sp. and *Cryptococcus* sp. to zinc toxicity in this study is similar to the report of Nweke and Okpokwasili (2010). Mahgoub *et al*., (2015) reported high dose inhibitory effect of zinc ion on *Candida* sp. Anochie and Ike (2016) reported that there was consistency in toxicity trend with increasing cadmium concentrations and was chosen to be best suitable for the dehydrogenase assay following the consistent pattern of toxicity of increasing cadmium concentrations to *Bacillus* species.

Toxic index was applied on the data to explore the possible mechanism of the interactions of the chemicals. The chemicals in the mixtures produced synergistic, additive and antagonistic effect. Antagonistic effect of zinc and cadmium recorded in this study presumes that zinc may reduce the toxicity of cadmium on dehydrogenase (enzyme) activity. Antagonistic interaction was observed by toxicity of zinc and cadmium binary mixtures on sea urchin embryo, while additive interaction was observe in quaternary mixtures of copper, lead, zinc and cadmium (Xu *et al*., 2011). Quaternary mixtures of nonessential metals exhibited synergistic interaction (Ishaque *et al.,* 2006). The toxic index model used to analyze binary mixtures toxicity indicated similar results as regards the toxicity of zinc, phenol and zinc, cadmium mixtures against dehydrogenase activity of the yeasts evaluated in this study. There observed synergistic and antagonistic responses to the joint action of the mixtures. The toxic interactions of the ternary mixtures were all observed

to be synergistic interaction. In this study, the order of toxicity zinc>phenol is in line what is reported in Nweke and Okpokwasili (2014). The order of toxicity is cadmium>zinc>phenol is in line with the report of Nwanyanwu *et al.,* (2017), in their work combined effects of metals and chlorophenols on dehydrogenase activity of bacterial consortium.

CHAPTER FIVE

5.0 CONCULSION AND RECOMMENDATION

5.1 Conclusion

In the present study, the phenol degrading yeast *Saprochaete* sp. and *Cryptococcus* sp. were isolated from hydrocarbon impacted soil. Investigated cultures of *Saprochaete* sp. and *Cryptococcus* sp. were able to eliminate almost entirely the introduced quantity (1000 mg/l) of phenol as sole source of carbon and energy for a period of 3 to14 days. *Saprochaete* sp. and *Cryptococcus* sp. have high tolerance for hydrocarbons like petrol, kerosene, phenol, diesel and crude oil when supplemented with mineral salt broth.

The results from the study also demonstrated that cadmium is more toxic to both *Saprochaete* sp. and *Cryptococcus* sp. than zinc and phenol, while phenol is the least toxic. Cadmium is one of the most toxic and non-essential heavy metals. However, the mechanisms underlying the toxic effects are still largely unknown. Toxicity of nonessential metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions. For example, lead (Hg^{2+}), cadmium (Cd^{2+}) and silver (Ag^{2+}) tend to bind to sulfhydryl (−SH) groups of enzymes essential for microbial metabolism, and thus inhibit the activity of sensitive enzymes. It can also be due to its chemical similarity to certain essential mineral elements, example zinc (Zn), iron (Fe) and calcium (Ca). Cadmium toxicity rises from displacement of these essential elements from a number of essential metalloproteins. Some heavy metals like copper, nickel, zinc and cobalt are essential trace elements required for normal physiological function of microorganisms, while others like cadmium and mercury have no known physiological function and are toxic even at low concentrations. At high concentrations, both essential and non-essential metal are toxic to organisms. Zinc exhibited more toxic effect than phenol. Phenol is less toxic because organisms use it as carbon source. However, at low doses, phenol showed stimulatory effect on the enzyme activity. Zinc is more toxic because at higher concentrations these heavy metal ions form unspecific complex compounds within the cell, which leads to toxic effects, making them too dangerous for any physiological function. To have a physiological or toxic effect, most

metal ions have to enter the microbial cell. Many divalent heavy metal cations (for example, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}) are structurally very similar

The analysis with toxic index model indicated that in most of the binary combinations, the interactions were synergistic and antagonistic, while that of ternary mixtures are all synergistic.

5.2 Recommendations

The present finding will be useful to treat the pollutants containing phenol to convert the toxicant into nutrient, biomass and CO_2 via biodegradation through their intermediates. This technology will be useful to the government, mechanic workshops and petroleum refineries which generate the waste containing compounds such as phenols.

Nevertheless, this study revealed that *Saprochaete* sp. and *Cryptococcus* sp. degrade phenol and also increase in concentrations of phenol, zinc and cadmium can in turn decrease the dehydrogenase (enzyme) activity of these yeasts, thereby inhibiting the growth of the yeasts. Therefore, care should be taken to avoid increase exposure of these toxicants to the soil microbial community, either in their individual or combined state as it is very toxic and can harm the useful microbial populations in the soil. This study will also serve as a guideline for industries so as to know the concentration at which the chemicals released into the environment can be toxic to microbial population in the soil. The effect of phenols and heavy metals should be taken into consideration in the risk assessment of heavy metals and phenol pollution of both agricultural and aquatic environment.

Saprochaeta sp and *Cryptococcus* sp present a great potential for the biodegradation of phenol and possibly of other related phenolic or aromatic compounds. Such microorganisms can be further studied for use in industrial effluent treatment and decontamination of natural areas.

REFERENCES

Abd El-Zaher, E.H.F., Mahmoud, Y.A.G.1 and Aly, M.M. (2011). Effect of different concentrations of phenol on growth of some fungi isolated from contaminated soil. *African Journal of Biotechnology*, **10**(8): 1384-1392.

Agency for Toxic Substances and Disease Registry (ATSDR). (1990). Toxicological Profile for Silver; U.S. Department of Health and Human Services, Public Health Service: Atlanta, GA, USA.

Agarry, S.E., Solomon, B.O. and Audu, T.O.K. (2010). Substrate utilization and inhibition kinetics: Batch degradation of phenol by indigenous monoculture of *Pseudomonas aeruginosa*. *International Journal for Biotechnology and Molecular Biology, Research*, **1**(2): 22 – 30.

AltTox.org. (2012). Toxicity Endpoints and Tests.

Amber, D. (2008). Soil ecology: What lies beneath. *Nature*, 455 (7214): 724–5.

Amorati, R. and Valgimigli, L. (2012). Modulation of the antioxidant activity of phenols by non-covalent interactions. *Organic and Biomolecular Chemistry*, **10** (21): 4147–58.

Andersen, A. A. (1958). New sampler for the collection, sizing, and enumeration of viable airborne particles. *Journal of Bacteriology,* **76**:471-484.

Anochie, C.C. and Ike, C.C. (2016). Effect of concentration of growth media on cadmium toxicity to isolated river water bacteria (*Bacillus species*). *International Journal of Scientific Engineering and Applied Science (IJSEAS),* 2:2395-3470.

Arendrup, M.C., Boekhout. T., Akova, M., Meis, J.F., Cornely, O.A. and Lortholary, O. (2014). ESCMID and ECMM joint clinical guidelines for the diagnosis and management of rare invasive yeast infections. *Clinical Microbiology Infection*, **20**(3):76–98.

Atagana, H. and World J. (2000). *Microbial Biotechnology,* **20**: 845-849.

Austin, M. (1953). The Skin of the Earth (1st ed.). London, UK

Azza, A., Abou, Z., Wesam, A.H., Hedayat, M.S., Ghada, A. and Fahd, A. (2009). Biosorption of some heavy metal ions using bacterial species isolated from agriculture waste water drains in Egypt. *Journal Applied Science Research*, **5**(4): 372-383.

Bahi, J. S., Radziah, O., Samsuri, A. W., Aminudin, H. and Fardin, S. (2012). Bioleaching of heavy metals from mine tailings by *Aspergillus fumigatus. Bioremediation Journal,* **16**:57-65.

BioWiki. (2016). Oxidative Reactions: Dehydrogenase and Oxidases . *biowiki.ucdavis.edu*.

Boillot, C. and Perrodin, Y. (2008). Joint-action ecotoxicity of binary mixtures of glutaraldehyde and surfactants used in hospitals: use of the Toxicity Index model and isobologram representation. *Ecotoxicology Environmental Safety,* **71**:252 – 259.

Bouyoucos, G.J. (1962). Hydrometer method improved for making particle size analysis of soils. *Agronomy Journal,* **54**:464-465.; Standards Association of Australia. AS 1289.C6.2-1976. Determination of the Particle Size Distribution of a Soil: An Analysis by Sieving in Combination with Hydrometer Analysis.

Bruins M.R., Kapil S. and Oehme F.W. (2000). Microbial resistance to metals in the environment. *Ecotoxicology Environmental Safety*, **45**:198–207.

Bunting, L. A., J. B. Neilson, and G. S. Bulmer. (1979). Cryptococcus neoformans: gastronomic delight of a soil ameba. *Sabouraudia,* **17**:225-232.

Casadevall, A. and Perfect, J.R. (1998) *Cryptococcus neoformans*. *American Society for Microbiol*ogy, ASM Press, Washington DC, 1st edition.

Calabrese, E. J. (2008). Hormesis: why it is important to toxicology and toxicologists. *Environmental Toxicology and Chemstry*, **27**:1451–1474

Campbell, C.K., Johnson, E.M. and Warnock, D.W. (2013). Identification of Pathogenic Fungi (2nd ed.). West Sussex, U.K: Blackwell Publishing Ltd, 263-304.

Chen, K.N., Chen, M.J. and Lin, C.W. (2006). Optimal combination of the encapsulating materials for probiotic microcapsules and its experimental verification (R 1). *Journal Food Engineering,* **76**: 313-322.

Chesworth, W. (2008). Dordrecht, the Netherlands: *Springer*.

Choudhury, R. and Srivastava, S. (2001). Zinc resistance mechanisms in bacteria. *Current Science,* **81**(70): 768-775

Colwell, R.R. and Walker, J.D. (1977). Ecological aspects of microbial degradation of petroleum in the marine environment. *Critical Reviews Microbiology,* **5**(4): 423 - 445.

Cravotta, I. C. A. and Brady, K. B. C. (2015). Priority pollutants and associated constituents in untreated and treated discharges from coal mining or processing facilities in Pennsylvania, USA. *Applied Geochemistry,* **62**:108-130

Damisa, D., Oyegoke, T.S., Ijah, U.J.J., Adabara, N.U., Bala, J.D. and Abdulsalam, R. (2013). Biodegradation of Petroleum by fungi isolated from unpolluted tropical soil. *International Journal of Applied Biology and Pharmaceutical Technology*, **4**(2):136-140.

Dey, S. and Mukherjee, S. (2010). Performance and kinetic evaluation of phenol biodegradation by mixed microbial culture in a batch reactor. *International Journal of Water Resources and Environmental Engineering*, **2**(3):40-49.

Doelman, P., Jansen, E., Michels, M. and Til, M. (1994). Effects of heavy metals in soil on microbial diversity and activity as shown by the sensitivity- resistance index, an ecologically relevant parameter. *Biology Fertility Soils*, **17**: 177–1784.

Donald, V. (2006). Fundamentals of Biochemistry: *Life at the Molecular Level*. New York: Wiley.

El.Deeb, B. and Altalhi, D. (2009). Degradative plasmid and heavy metal resistance plasmid naturally coexist in phenol and cyanide assimilating bacteria. *American Journal of Biochemistry Biotechology,* **5**(2): 84-93.

EL-Mahi, Y.E., Ibrahim, I.S., Abel-Majid, H.M. and Eltilib, A.M.A. (1987). A simple method for the estimation of calcium and magnesium carbonates in soils. *Soil Society of America Journal,* **51**(5): 1152- 1155.

El-Nahash, M.H., Al-Muhtaseb, S.A. and Makhlouf, S. (2009). Biodegradation of phenol *Pseudomonas putida* immobilized in poly vinyl alcohol (PVA) gel. *Journal Hazardous Materials,* **164**(2-3):720-725

Emmons, C. W. (1955). Saprophytic sources of *Cryptococcus neoformans* associated with the pigeon (Columbalivia). *American Journal of Hygiene,* **62**:277-232.

Emmons, C. W. (1951). Isolation of *Cryptococcus neoformans* from soil. *Jounal of Bacteriology*, **62**:685-689.

Environmental Protection Agency (EPA) (2002). Toxicological Review of phenol. CSA No. 108-95-2

Francek, M. A., Makimaa, B., Pan, V and Hanko, J. H. (1994). Small town Lead levels: A case study from the homes of Pre – Schooler in MT – Pleasant, Michigan. *Environromental Pollution*, 0269 – 7491/94/307.00 © Elsevier Science Limited, England, 159-167.

Gabrielson, J., Hart, M., Jarelov, A., Kuhu, I., Mckenzie, D. and Mollby R. (2002). Evaluation of redox indicators and the use of digital scanner and spectrophotometers for quantification of microbial growth in microplate. *Journal of Microbiological Methods,* **50**:63-73.

Gaudette, H., Flight, W., Toner, L. and Folger, D. (1974). An in expensive titration method for the determination of organic carbon in recent sediments. *Journal of Sedimentary Petrology*, **44**:249-253.

Gikas P. (2007). Kinetic responses of activated sludge to individual and joint nickel (Ni(II)) and cobalt (Co(II)): An isobolographic approach. *Journal of Hazardous Materials*, **143**:246–256.

Girma, G. (2015). Microbial Bioremediation of some Heavy Metals in Soils: An updated review. *Journal of Resources Development and Management*, **10**:62-73

Groten, J.P., Feron, V.J. and Suhnel, J. (2001). Toxicology of simple and complex mixtures. *Trends in pharmacological Science,* **22**: 316-322.

Husaini, A., Roslan, H.A., Hii, K.S.Y. and Ang, C.H. (2008) Biodegradation of aliphatic hydrocarbon by indigenous fungi isolated from used motor oil contaminated sites. *World Journal of Microbiology and Biotechnol*ogy, **24**: 2789-2797.

Hättenschwiler, S. and Vitousek, P. M. (2000). The role of polyphenols in terrestrial ecosystem nutrient cycling. *Trends in Ecology and Evolution*, **15** (6): 238–243.

Heipieper, H.J., Diefenbach, R. and Keweloh, H. (1992). Conversion of cis unsaturated fatty acids to trans, a possible mechanism for the protection of phenol degrading *Pseudomonas putida* P8 from substrate toxicity. *Applied Environental Microbiology,* **58**: 1847 – 1852.

Hong, K. J., Tokunaga, S. and Kajiuchi, T. (2002). Evaluation of Remediation Process with Plant-Derived Bio-surfactant for Recovery of Heavy Metals from Contaminated Soils. *Chemosphere*, 49(4): 379-387.

Haldane, J. B. S. and Briggs, G. E. (1925). A note on the kinetics of enzyme action. *Biochemical J*ournal, **19**: 338–339.

Liu, H., Yu, Q.J., Wang, G., Ye, F. and Cong, Y. (2011). Biodegradation of phenol at high concentration by a novel yeast *Trichosporon montevideense* PHE1. *Process Biochem*istry, 46:1678–1681.

Inuwa, M., Abdulrahman, F.W., Bimin Yauri, U.A. and Ibrahim, S.A. (2007). Analytical Assessment of Some Trace Metals in Soils around the Major Industrial Areas of Northern Nigeria. *Trend in Applied Science Research*, **2**(6):515-521.

Iram, S., Uzma, G., Rukh, S. and Ara, T. (2013) Bioremediation of Heavy Metals Using Isolates of Filamentous Fungus *Aspergillus fumigatus* Collected from Polluted Soil of Kasur, Pakistan, *International Research Journal of Biological Sci*ence, 2: 66-73.

Ishaque, A. B., Johnson, L., Gerald, T., Boucaud, D., Okoh, J. and Tchounwou, P. B. (2006). Assessment of individual and combined toxicities of four non-essential metals (As, Cd, Hg and Pb) in the Microtox Assay. *International Journal of Environmental Research and Public Health,* 3:118-120

Jayachandran, V.P. and Kunhi, A.A.M. (2008). Degradation of 3-chlorobenzoate and phenolsingly and in mixture by a mixed culture of two ortho pathways following *Pseudomonas* strains. *Journal Industrial Microbiology and Biotechnol*ogy, 5: 120-122.

Jin, H.P., Dane, L., Periyasamy, P., Girish, C., Nanthi, B. and Jae-Woo, C. (2011). Role of organic amendments on enhanced bioremediation of heavy metal (loid) contaminated soils, *Journal of Hazardous Materials*, **185**: 549–574.

Kabata-Pendias, A. (2000). Trace Elements in Soils and Plants. *CRC Press*, Boca Raton, USA.

Karimi, M and Hassanshahian, M. (2016). Isolation and characterization of phenol degrading yeasts from wastewater in the coking plant of Zarand, Kerman. *Braz. J. Microbiol.*, **47**(1): 18–24.

Karimi, M. and Hassanshahian, M. (2016). Isolation and characterization of phenol degrading yeasts from wastewater in the coking plant of Zarand, Kerman. *Brazilian Journal of Microbiol*ogy, **47**(1): 18-24.

Kavita, V. and Palanivelu, K. (2004). The role of ferrous ion in Fenton and photo Fenton process for the degradation of phenol. *Chemosphere*, **55**:1235-1243.

Kim, S. (1985). Effect of heavy metals on natural populations of bacteria from surface microlayers and subsurface water. *Marine Ecology Program Series,* **26**: 203-206.

Kotresha, D. and Vidyasagar, G.M. (2008). Isolation and characterisation of phenol-degrading *Pseudomonas aeruginosa* MTCC 4996. *World Journal of Microbiology and Biotechnology*, **24**(1): 541-547.

Lee L. H. and Lustigman B. (1996). Effects of barium and nickel on the growth of Anacystis nidulans. *Bulletin of Environ. Contamination and Toxicology,* **56**:985–992.

Leonard, D. and Lindley, N.D. (1999). Growth of *Ralstonia eutropha* on inhibitory concentrations of phenol: diminished growth can be attributed to hydrophobic perturbation of phenol hydroxylase activity. *Enzyme and Microbial Technology,* **25**:271– 277.

Loganathan, P. (1984). Laboratory Manual of Soil and Plant Analysis. Rivers State University of Science and Technology, Port- Harcourt, Nigeria.

Mac Gillivray, A. and Shiaris, M. (1993). *Applied Environmental Microbiology*, **59**:1613-1618.

Marianna, A.V. and Anna Maria, T. (2009). *Cryptococcus*. *Clinical Mycology,* (2nd edition). 231

Mahgoub, S., Abdelbasit, H. and Abdeifattah, H. (2015). Removal of phenol and zinc by *Candida* isolated from wastewater for integrated biological treatment. *Desalination and Water Treatment,* **53**: 3381-3387.

Matsumura, Y. and Ananthaswamy, H.N. (2004). Toxic effects of ultraviolet radiation on the skin. *Toxicology and Applied Pharmacoloy*, **195**: 298–308.

Mazzocato, S., Marchionni, E., Fothergill, A.W., Sutton, D.A., Staffolani, S. and Gesuita, R. (2014). Epidemiology and outcome of systemic infections due to *Saprochaete capitata*: case report and review of the literature. *Infection*. 43(2):211–15.

McLean, E. O. (1982). Soil pH and lime requirement. *Methods of soil analysis. Part 2. Chemical microbiological properties*, (methodsofsoilan2), 199-224.

Meghraj H. and Daneshwar P. (2013). Isolation and Identification of Heavy Metals Tolerant Bacteria from Industrial and Agricultural Areas in Mauritius, *Current Research in Microbiology and Biotechnology*, **1**(3) : 119-123.

Merriam-Webster Dictionary. (1828). Toxicity.

Monod, J. (1949). The growth of bacterial cultures. *Annual Review Microbiology*, **3**: 371–394.

Morrison, R.M. and Boyd, R.N. (1992). *Organic chemistry*, 6th ed. 1069-1098.

Neha, S. and Vikas, C. G. (2012). Batch Biodegradation of Phenol of Paper and Pulp Effluent by *Aspergillus Niger. International Journal of Chemical Engineering and Applications,* **3**(3): 182-186.

Neumaan, G., Teras, R., Manson, L., Kivisaar, M., Schayer, F. and Heipieper, H.J. (2004). Simultaneous degradation of atrazine and phenol by *Pseudomons* sp. ADP: Effects of toxicity and adaptation. *Applied Environental Microbiology,* **70**:1907-1912.

Nies D. H. (1999). Microbial heavy-metal resistance. *Applied Microbiology and Biotechnology*, **5**:730–750.

Nortcliff, S., Hulpke, H., Bannick, C.G., Terytze, K., Knoop, G., Bredemeier, M. and Schulte-Bisping, H. (2012). Soil, 1. Definition, Function, and Utilization of Soil. *Ullmann's Encyclopedia of Industrial Chemistry,* 33:400-418.

Nwanyanwu, C.E., Adieze, I.E.,Nweke, C.O. and Nzeh, B.C. (2017). Combined effects of metals and chlorophenols on dehydrogenase activity of bacterial consortium. *International Research Journal of Biological Sciences,* **6**(4):10-20

Nwanyanwu, C. E. and Abu, G.O. (2013). Biodegradation of phenol at low and high doses by bacterial strains indigenous to Okrika River in the Niger Delta of Nigeria. *Journal of Research in Biology,* **3**(3): 911-921.

Nwanyanwu, C. E. and Abu, G.O. (2012). Growth and degradation responses of phenol-utilizing bacteria to increased doses of phenol in petroleum refinery waste water. *Internatioal Journal of Bioscience (IJB),* **2**(10):125-134.

Nweke, C.O., Ntinugwa, C., Obah, I.F., Ike, S.C., Eme, G.E., Opara, E.C., Okolo, J.C. and Nwanyanwu, C.E. (2007). *In vitro* effects of metals and pesticides on dehydrogenase activity in microbial community of cowpea (*Vigna unguiculata* authority) rhizoplane. *African Journal Biotechnology,* **6**(3): 290-295.

Nweke, C.O. and Okpokwasili, G.C. (2010). Influence of exposure time on phenol toxicity to refinery wastewater bacteria. *Journal of Environmental Chemical and Ecotoxicology,* **2**:20-27.

Nweke, C.O. and Okpokwasili, G.C. (2014). Kinetics of growth and phenol degradation by *Pseudomonas* species isolated from petroleum refinery wastewater. *International Journal of Bioscience (IJB),* **4**(7):28-37.

Nweke, C.O., Ike, C.C. and Ibegbulem, C.O. (2016). Toxicity of quaternary mixtures of phenolic compounds and formulated glyphosate to microbial community of river water. *Ecotoxicoloy and Environmental Contam*ination, **11**(1): 63-71.

Oboirien, B.O., Amigun, B., Ojumu, T.V., Ogunkunle, O.A., Adetunji, O.A., Betiku, E. and Solomon, B.O. (2005). Substrate inhibition kinetics of phenol degradation by *Pseudomonas aeruginosa* and *Pseudomonas fluorescence*. *Biotechnology*, 4(1):56 – 61.

Odu, C.T.I., Babalola, O., Udo, E.J., Ogunkunle, A.O., Bakare, T.A. and Adeoye, G.O. (1986). Laboratory manual for agronomic studies in soils, plants and microbiology. Dept of Agronomy, Univ. of Ibadan. 83.

Okpokwasili, G.C. and Nweke, C.O. (2006). Microbial growth and substrate utilization kinetics. *African Journal of Biotechnology,* **5**(4): 305 -317.

Okpokwasili, G. C. and Okorie, B. B. (1988). Biodeterioration potentials of microorganisms isolated from car engine lubricating oil. *Tribology International,* 21 (4):215 220.

Okolo, J. C., Nweke C. O., Nwabueze R. N., Dike C. U. and Nwanyanwu C. E. (2007). Toxicity of phenolic compounds to oxidoreductases of *Acinetobacter* species isolated from a tropical soil. *Scientific Research Essay,* **2**:244–250.

Olsen, S.R. and Sommers, L.E. (1982) Phosphorus. In: Page, A.L., Ed., Methods of Soil Analysis Part 2 Chemical and Microbiological Properties, *American Society of Agronomy*, *Soil Science Society of America*, *Madison*, 403-430.

Pamidimukkala, U., Kancharla, A., Sudhaharan, S., Gundeti, S., Mandarapu, S., Nagalla, V.K., Raju, S.B., and Karanam, S.D. (2017). Isolation of the Rare Opportunistic Yeast *Saprochaete capitata* from Clinical Samples-Experience from a Tertiary Care Hospital in Southern India and a Brief Review of the Literature. *Journal of Clinical Diagnostic Research,* 11(9): DC36–DC42.

Ponge, J. (2015). The soil as an ecosystem. *Biology and Fertility of Soils,* **51** (6): 645–648.

Preston, S., Coad, N., Townend, J., Killham, K. and Paton, G.I. (2000). Biosensing the actue toxicity of metal interactions: are they additive, synergistic, or anatagonistic? *Environmental Toxicology and Chemistry*, **19**:775-780.

Ren, S. and Frymier, P.D. (2003). Toxicity estimation of phenolic compounds by bioluminescent bacterium. *Journal of Environmental Engineering (ASCE),* **129**: 328- 335.

Reuters, T. (2015). Toxicology. *Journal Citation Reports.* Web of Science *(Science ed.).*

Rigo, M. and Alegre, R. (2004). Isolation and Selection of Phenol degrading microorganism from industrial wastewater and kinetics of the biodegradation, *Folia Microbiology,* **49** (1): 41-45.

Riquelme, J.M., Correa, V., Araya, P., Neira, M. and Yévenes, I. (2015). Water and Physiological Saline to Prevent the Formation of P-Chloroaniline. *International Journal Odontostomat*ology, **9**(3):399-404.

Roesch, L.F., Fulthorpe, R.R., Rivam, A., Casella, G., Hadwin, A.K., Kent, A.D., Daroub, S.H., Camargo, F.A., Farmerie, W.G. and Triplett, E.W. (2007). "Pyrosequencing enumerates and contrasts soil microbial diversity". *The ISME Journal,* **1** (4): 283–90.

Ross, A. and Taylor, I.E. (1981.) Extracellular glycoprotein from virulent and avirulent *Cryptococcus* species. *Infection and Immunity*. **31**(3):911–8.

Salem, H.M., Eweida, E.A. and Farag, A. (2000). Heavy metals in drinking water and their environmental impact on human health. In *ICEHM*; Cairo University: Giza, Egypt, 542– 556.

Sandhu, S. S., Shakya, M., Deshmukh, L., Aharwal, P.R. and Kumar, S. (2016). Determination of hydrocarbon degrading potentiality of indigenous fungal isolates. *International Journal of Environmental Science,* **6**(6): 1163-1172.

Santos, L.V. and Valter R. Linardi, V.R. (2001). Phenol degradation by yeasts isolated from industrial effluents. *Journal of General and Applied Microbiology*, **47**: 213–221.

Santo, C. E., Vilar, V. J.P., Botelho, C. M. S., Bhatnagar, A., Kumar, E. and Boaventura, R. A.R. (2012). Optimization of coagulation-flocculation and flotation parameters for the treatment of a petroleum refinery effluent from a Portuguese plant. *Chemical Engineering Journal,*183: 133-13.

Sani R. K., Peyton B. M. and Jandhyala M. (2003). Toxicity of lead to Desulfovibrio desulfuricans G20. *Environmental Toxicology and Chemistry,* **22**:252–260.

Seker, S., Beyenal, H., Salih, B. and Tomas, A. (1997). *Appl. Microbiol. Biotechnol*. **47**: 610- 614.

Sikkema, J., De Bont, J.A.M. and Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. *Microbiology and Molecular Biology Reviews*, **59**: 201-222

Smadar, L. and Mordechai, C. (2009). Synergistic cytotoxic effect of tetrachlorocatechol and sodium amide in *Echerichia coli*: toxicity, metabolism, and mechanistic aspect. *Environmental Toxicology and Chemistry,* **28**: 1380-1389

Šmejkalová, M., Mikanová, O. and Bor_ka, L. (2003). Effects of heavy metal concentrations on biological activity of soil microorganisms. *Plant Soil and Enviroment*, **49**(7): 321-326.

Stewart, E. A., Grimshaw, H. M., Parkinson, J. A. and Quarmby, C., (1974). *Chemical Analysis of Ecological Materials*. Blackwell Publications. London

Supriya, C. and Neehar, D. (2014). Biodegradation of phenol by *Aspergillus niger. Journal of Pharmacy,* 4(7):11-17.

Suranjana, R. and Manas, K. R. (2009). Bioremediation of Heavy metals Toxicity – with special Reference to Chromium, *Special*: 57-63

Tanee, F.B.G. and Albert**,** E. (2015). Reconnaissance Assessment of Long-Term Effects of Crude Oil Spill on Soil Chemical Properties and Plant Composition at Kwawa, Ogoni, Nigeria**.** *Journal of Environmental Science and Technology,* **8**(6): 320-329

Teixeira, L. A. C., Nildo de Abreu V. J., Lidia Y. and Fabiana V. (2015). Degradation of phenol in mine waters using hydrogen peroxide and commercial steel wool. *International Journal of Mineral Processing,* **138**: 15-19.

Tziotzios, G., Teliou, M., Kaltsouni, V., Lyberatos, G. and Vayenas, D. V. (2005). Biological phenol removal using suspended growth and packed bed reactors. *Biochemical Engineering Journal* **26**(1): 65- 71.

Umabala, P., Anuradha, K., Sukanya, S., Sadashivudu, G., Surendra, M., Vamsi, K.N., Sree, B.R. and Sandhya, D.K. (2017). Isolation of the Rare Opportunistic Yeast *Saprochaete capitata* from Clinical

Samples-Experience from a Tertiary Care Hospital in Southern India and a Brief Review of the Literature. *Jornal of Clinical and Diagnostic Research*, **11**(9): DC36–DC42.

Uzukwu, P.U., George, O.S. and Jamabo, N.A. (2011). The Problem of Water Seepage in Aquaculture: A Preliminary Study of the Soils of Arac Fish Farm, Omuihuechi-Aluu, Rivers State, Nigeria. *Asian Journal of Agricultural Sciences,* **3**(2): 63-69.

Varma, R.J. and Gaikwad, B.G. (2009). Biodegradation and phenol tolerance by recycled cells of *Candida tropicalis* NCIM 3556. *International Biodeterioration and Biodegradation,* **63**(4): 539-542.

Voroney, R. P. and Heck, R. J. (2015). The soil habitat. In Paul, Eldor A. *Soil Microbiology, Ecology and Biochemistry* (4th ed.). Amsterdam.

Walkley, A. and Black, I.A. (1934). An examination of the Degtjareff method for determining of the organic matter and the proposed modification of the chromic acid titration method. *Soil Science*, **37**:29-38.

Wang, D., Zhong, W. and Xu, X. (2012). Phenol removal efficiencies of sewage treatment processes and ecological risks associated with phenols in effluents. *Research Center for Eco-Environmental Science CASS.* **286**: 217-218.

Wasiullah, R.D., Malaviya, D., Pandiyan, K., Singh, U.B., Sahu , A., Shukla, R., Singh, B.P., Rai, J.P., Sharma, P.K., Lade, H. and Paul, D. (2015). Bioremediation of Heavy Metals from Soil and Aquatic Environment: An Overview of Principles and Criteria of Fundamental Processes. *Sustainability*, **(7)**: 2189-2212.

Watanabe, K., and Teramoto, H., Futamata, H. and Harayama, S. (1998). *Applied Environmental Microbiology,* **64**: 4396-4402.

WHO. (1994). Phenol, Environmental Health Criteria-EHC 161, World Health Organization, Geneva.

Wuana, R.A. and Okieimen, F.E. (2011). Heavy metals in contaminated soils: A review of sources, chemistry, risks and best available strategies for remediation. *Ecology,* **20**.

Xu, X., Li, Y., Wang, Y. and Wang, Y. (2011). Assessment of toxic interactions of heavy metals in multi-components mixtures using sea urchin embryo-larval bioassay. *Toxicology in Vitro*, **25**:294-30.

Zhang, X. and Wiegel, J. (1990). Sequential anaerobic degradation of 2, 4 dichlorophenol in freshwater sediments. *Applied Environmental Microbiology*, **56**: 1119-1127.

APPENDIX I

Composition of nutrient broth

Components	Mass (g)
Peptone	5.0
Beef extract	1.0
Yeast extract	2.0
NaCl	5.0
Distilled water	1000ml

Composition of nutrient agar

Components	Mass (g)
Peptone	5.0
Beef extract	1.0
Yeast extract	2.0
NaCl	5.0
Agar	15.0
Distilled water	1000ml

Composition of Potato Dextrose Agar (PDA)

Components	Mass (g)
Potato	200
Dextrose	20
Agar	20
Distilled water	1000ml

Composition of Mineral Salt Medium (MSM)

Components	Mass (g)
NaCl	10.0
$MgSO_4.7H_2O$	0.42
KCl	0.29
KH_2PO_4	0.83
Na_2HPO_4	1.25
$NaNO_3$	0.42
Agar	20.0
Distilled water	1000ml

Composition of Mineral Salt Broth (MSB)

Components	Mass (g)
NaCl	10.0
$MgSO_4.7H_2O$	0.42
KCl	0.29
KH_2PO_4	0.83
Na_2HPO_4	1.25
$NaNO_3$	0.42
Distilled water	1000ml

APPENDIX II

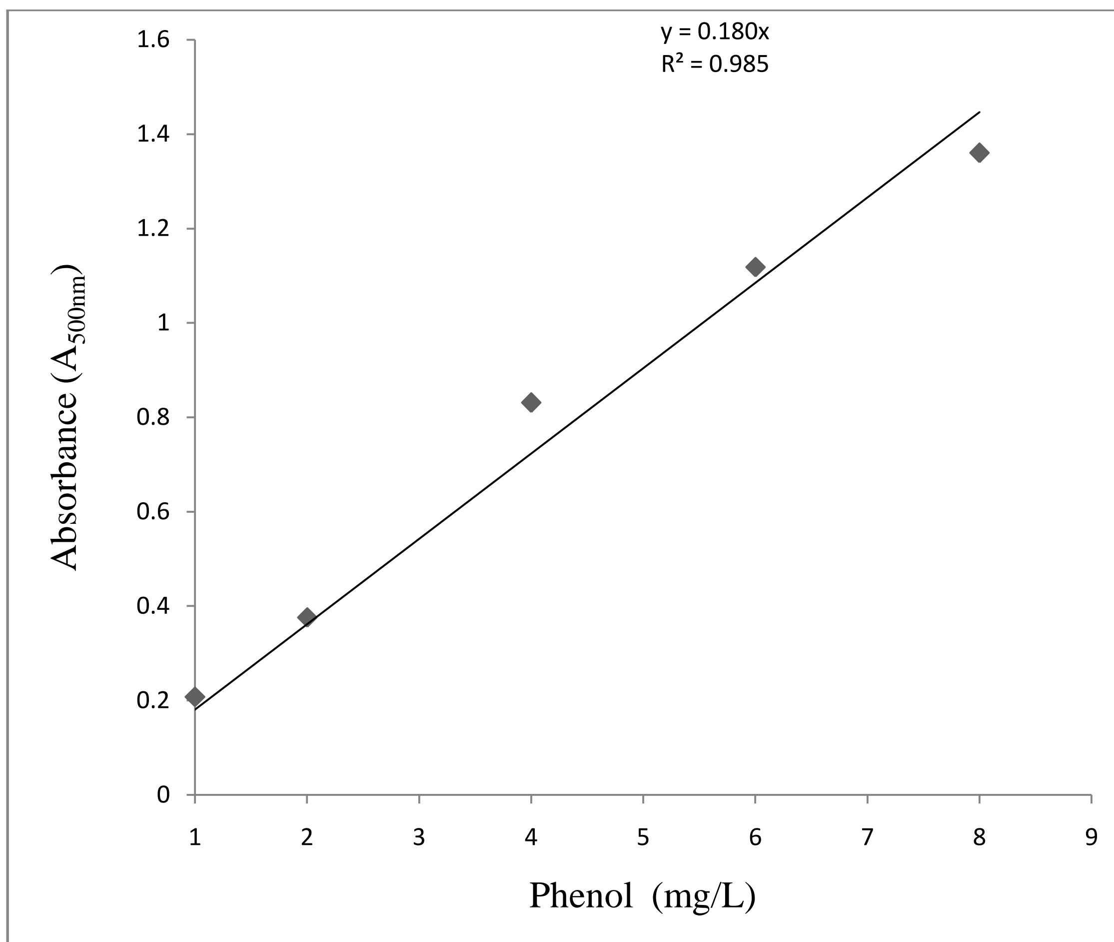


Figure 1: Phenol calibration curve.

APPENDIX III

t-Test: Two-Sample Assuming Unequal Variances

	0.385	*0.358*
Mean	1.225	1.184588235
Variance	2.03314525	2.268491757
Observations	17	17
Hypothesized Mean Difference	0	
df	32	
t Stat	0.080336961	
P(T<=t) one-tail	0.468234801	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	0.936469603	
t Critical two-tail	2.036933334	

There is no significant difference (0.0803) in the IC_{50} of both organisms at 0.5% significant level.