Degradation of Phenol by Using Consortium of Selected Microbial Strains

By

TAYYABA ASHFAQ

(Reg#00000206745)

A thesis submitted in partial fulfillment of requirements for the degree of

Master of Science

In

Environmental Science

Institute of Environmental Sciences and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

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Islamabad, Pakistan

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It is certified that the contents and form of the thesis entitled

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Submitted by

Ms. Tayyaba Ashfaq

Has been found satisfactory for partial fulfillment of the requirements of the degree of

Master of Science in Environmental Science

Supervisor: _____

Dr. Muhammad Arshad

Associate Professor

IESE, SCEE, NUST

Member: _____

Dr. Imran Hashmi

Professor

IESE, SCEE, NUST

Member: _____

Dr. Deedar Nabi

Assistant Professor

IESE, SCEE, NUST

THESIS ACCEPTANCE CERTIFICATE

Certified that final copy of MS thesis written by **Ms. Tayyaba Ashfaq** (Registration No. 00000206745) of **IESE (SCEE)** has been vetted by the undersigned, found complete in all aspects as per NUST Statutes/Regulations, is free of plagiarism, errors and mistakes and is accepted as partial fulfilment for award of MS Degree in Environmental Sciences. It is further certified that necessary amendments as pointed out by GEC members of the student have been also incorporated in the said thesis.

Signature: _____

Name of Supervisor: Dr. Muhammad Arshad

Date: _____

Signature of HoD: _____

Date: _____

Countersigned by

Signature (Dean/ Principal): _____

Date: _____

This thesis is dedicated to my Dada Abu and my Parents whose continuous support and prayers are always with me whenever and wherever required.

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Summary

Human activities and agricultural interventions enhance the input of toxic contaminants in water like petroleum hydrocarbons, insecticides and pesticides. Composition of hydrocarbon includes resin, asphaltene, aromatic and saturated hydrocarbon. This study mainly focused on phenol, because of its aromatic nature and solubility in water. Phenols are carcinogenic in nature and can cause many toxic health effects if its concentration increases above 5 µg/L. This study was performed to develop an integrated method for the degradation of phenol. Different bioreactors were designed (Four in number) to compare the phenolic degradation in the presence of microbes, plants and inoculated plants. Ryegrass can help to degrade the hydrocarbons but cannot grow properly when concentration of hydrocarbons enriches. To increase the efficacy of ryegrass, consortium of two endophytes (*Pseudomonas putida* and *Pseudomonas aeruginosa*) was employed. Dosage of 250 mg/L of phenol was applied to each bioreactor and supervised its degradation after every 6 hours all the way through UV visible spectrophotometer. Application of selected endophytes consortium promoted the degradation of phenol. In addition, the consortium accelerated seedling emergence, promoted plant growth rate under adverse conditions. The results demonstrated capability of consortium for assisting plants to tolerate stress from phenol and to improve phytoremediation of phenolic pollutants. This research work can contribute to develop effective remediation methodologies for cleaning up of phenol contaminated sites thus improving the overall quality of life and environment.

Chapter 1

INTRODUCTION

1.1 Background

Anthropogenic activities lead to increased concentration of noxious pollutants like petroleum hydrocarbons, pesticides and insecticides in the environment. However, petroleum hydrocarbons are considered the most prevalent contaminants among others (Qi et al., 2017). Petroleum industry is the backbone of global economy; however, it has significant impact on natural resources from exploration till final utilization. According to estimates, the peak conventional production of shale and crude oil, natural gas liquids (NGLs) and sands oil was calculated in 2016 to be 92.2 million barrels per day (Fatima et al., 2016). Industrialization and excessive discharge of harmful pollutants resulted in contamination of soil, air and water resources. Diesel fuel contaminates soil and water and it is categorized as hydrocarbon pollutant (Gallego et al., 2001). Presence of gasoline hydrocarbons has adverse consequences on human health and also on plants (Meudec et al., 2007).

Useful products like kerosene, diesel and gasoline are produced after the processing of crude oil in an oil refinery (Gary and handwerk, 1984). The crude oil has to go through separation or conversion, one or both of these processes to separate fractions (Hengstebeck, 1959). Unfortunately, these processes produce several aqueous waste products.

Petroleum products such as diesel, gasoline, asphalts and natural gas are increasingly used now a days and several marine and terrestrial sites have been contaminated, either directly by effluents or spills, or indirectly by terrestrial runoff (Perelo, 2010). Hydrocarbons of petroleum composed of different percentages of short, medium, and long aliphatic (i.e., alkanes, alkenes), mono aromatics (e.g., BTEX as well as phenolic), polycyclic aromatic hydrocarbons (known as PAHs, such as naphthalene, phenanthrene, and pyrene) (Frick et al., 1999). Phenol and catechol are major group of pollutants because of their persistent nature and lies in mono-aromatic group. In comparison to other mono-aromatics, phenol is highly soluble in water, its solubility is 84.2g/L and it can cause severe health effects and environmental problems. If the hydrocarbons enter the food-chain, they can be dangerous since few of them are persistent in nature (Perelo, 2010).

1.2 Phenol

Phenol usage in many industries like pesticides, coke, pharmaceuticals and refineries is becoming a major Environmental concern. Phenolic compounds can move into the environment through natural processes, like degradation of tannins, humic substances and lignin (Jabrou, 2012). Phenols are soluble in water and high solubility in water make them acute toxic to biodiversity. Phenolic compounds are highly toxic and a minor amount can be dangerous for the aquatic and human life. So, there is a need to establish a strategy which is economical as well as efficient to degrade the compound. Phenols are

carcinogenic in nature and a very little amount of phenol can affect the taste of water. Chronic health effects of phenol in human include liver injury, difficulty in breathing, headache and vomiting (Yadav and Harjit, 2014). As described by Environmental Protection Agency of USA (US-EPA), concentrations of phenolic compounds should not exceed more than 0.5µg/L in drinking water.

1.3 Harmful effects of phenol

Volatile organic compounds can easily move into the atmosphere, so they can affect public health directly or indirectly. Phenols are carcinogenic in nature and can cause many toxic health effects if its concentration increased from 5µg/L (Nasreen, 2020). Short term exposure to phenolic compounds show potential toxicity to marine life in water systems (especially in confined regions) as well as toxic breathing hazards. Chronic exposure of phenol causes vomiting, alterations in liver functioning, headache, difficulty in swallowing and fainting, etc. These chemicals are intense eye, skin and mucous membrane irritants and may cause cancer. These chemicals persist in the environment due to their wide spread applications in industrial processes (Tapilatu et al., 2010).

When humans are exposed to such toxic compounds in the form of a mixture, it leads to genetic, neurological, excretory and respiratory system malfunctioning (Yadav and Harjit, 2014).

1.4 Phenol removal

Several treatment technologies were used which include dilution technique, physical methods, offsite treatment, advanced oxidation process, controlled incineration, washing and photocatalysis used for phenol removal. However, most treatment options are not cost effective or they produce some harmful sub-products which create other environmental problems (Xue et al., 2015). So, we must have to develop and use sustainable treatment techniques which is cost effective and eco-friendly. To achieve this objective, researchers are focusing on different treatment methods especially biological method, plants in association with microbes. Extensive research must be performed to understand the mechanism involved and this is the main debate among researchers and scientific society since last two decades (Scoma et al., 2016).

Bioremediation is most preferred biological method of degrading and transforming toxic pollutant in the environment into less harmful substances. The process involves natural practices for remediation of infected site. Microbes utilize these toxic compounds as a source of carbon and energy and breaking them down into less harmful substances (Azubuike et al., 2016)

Bioremediation of phenol happens in both groundwater and soil. On the other hand, it is observed to be slow particularly at higher concentration, which is lethal to microbiota. The use of acclimatized microbial community can allow fast degradation. Phenol is considered to be completely degraded in groundwater within a time span of 8 days which includes a lag phase of 3-4 days whereas bacterial community adapts to the specific growth conditions. When phenol

enters into the water, its removal rate can be faster or take some weeks which is dependent on different environmental conditions like temperature, availability of nutrients, pH and rate of acclimatization.

1.5 Problem statement

Phenol can be produced by both naturally and synthetically, but synthetic production of phenol is increasing multifold. A study was conducted in 2015, which depicted that assembly of phenolic compounds exceeds 10 million tons and expected to rise 12 million tons in 2021 (Saleem et al., 2019).

There are many methods that can be used to degrade the phenol concentration like volatilization, adsorption and photodecomposition (Kulkarni and Kaware, 2013). Among all these methods, phytoremediation is an eco-friendly method but plants can degrade less amount of pollutants so to increase its efficiency, endophytic microbes can be introduced along with plants (Pradeep et al., 2015). Microbes correlated with plants degraded phenol to detoxify by using degradative genes (Jin et al., 2019).

Keeping in view this background, we developed bioreactors considering the design of the wetlands employing *Lolium prenne* (ryegrass) as a standard plant in partnership with the consortium of two endophytic bacteria *(Pseudomonas putida* and *Pseudomonas aeruginosa*).

1.6 Study Objectives

The objectives of present study were:

i. To develop a bioreactor for treating phenol contaminated water
ii. Monitoring the bacterial potential for degradation of phenol under control conditions
iii. To examine the enrichment of endophytic bacteria in a lab-scale system for efficient treatment of phenol

Overall this research work is an initiative in developing remediation methodologies for cleaning up of phenol contaminated sites thus improving the overall quality of life and environment.

Chapter 2

Literature review

Petroleum is a mixture of many different chemical compounds. It comprises of simple compounds such as methane as well as some other very complex hydrocarbons. Unlike water, crude oil will not evaporate, when heated to boiling and held there for quite a time (Leffler, 1979). According to U.S. Energy Information Association (2009), crude oil is being used in many ways, petroleum products are being used by the world to move from one place to another and to merchandise by using gasoline and diesel in engines, as well as crude oil can be used to make crayons, plastics, CD‘s, bubble gum and ink (Gary and Handwerk, 1984).

2.1 Oil refinery

An oil refinery converts crude oil into useful products like petroleum, diesel and kerosene oil. Different parts of the crude oil are used to make a wide range of products that are being used by consumers, by using the process of separation at an oil refinery. Separation and conversion are two main processes in almost all petroleum refineries. During the process of separation, petroleum moves through a hot furnace in modern separation (Waddams, 1980). Liquids and vapors are then discharged into distillation towers, gasoline and liquid petroleum gas which are the lightest components, they rise above and condensed back to liquids in the distillation tower, medium weight compounds stay in the

middle and at the bottom, there are heavy liquids (Gary and Handwerk, 1984). Cracking uses pressure and heat to break complex hydrocarbons into simpler ones, and hence it is the most commonly used conversion technique. It has been used in the refinery process since very long (Tarafdar and Sinha, 2018).

2.2 Refinery wastewater

Separation of crude oil into gasoline and other useful products uses water (Sharma and Simsek, 2019). Large quantities of wastewater are produced, which are contaminated with hydrocarbons or heavy metals. The amount of wastewater production is specific for every refinery and depends upon different parameters like treatment and disposal methods (UNEP, 1987). Water containing hydrogen sulfide is called sour water and it is the main wastewater from petroleum refinery (Sharma and Simsek, 2019).

2.3 Hydrocarbon

Hydrocarbons are present in environment as gasses or tiny particles and they are composed of hydrogen and carbon. Crude oil contains hydrocarbons. They are released into the environment through diesel and petrol used in automobiles. Aromatic hydrocarbons are of main concern because they contaminate environment by polluting air, water and soil. Three major classes of aromatic hydrocarbons are (i) polycyclic aromatic hydrocarbons (PAHs), (ii) heterocyclic aromatic hydrocarbons, and (iii) alkyl PAHs (McGuinness and Dowling, 2009). Most petroleum hydrocarbons can be firmly adsorbed on the soil organic matter, trapped in the pores of the soil (Hutchinson et al., 2004), and

long-chain alkanes can become factors in the formation of oil slicks and oil films, leading to the blockage of water, soluble nutrients and gas exchange (Valentyne et al., 2018).

2.4 Aromatic hydrocarbons contamination

The main source of aromatic hydrocarbon's pollution is the gasoline spill from improperly maintained underground gasoline storage tanks. Aromatic hydrocarbons enter into the environment from leakage in pipelines, surface and underground spills and release from large gasoline facilities. Due to harmful effects of these chemicals on human health, the U.S. EPA has modified maximum allowable levels of these pollutants in water for human use (U.S. EPA, 2006). Once these compounds become part of the environment, they readily volatize, get attached to soil particles by getting into the soil pore places or biodegrade.

Evaporation happens when these hydrocarbons volatilize, which allows them to get into the atmosphere causing air pollution. Evaporation of the aromatic compounds takes place when gasoline is being pumped in the car causing characteristic smell. The same process occurs within the air spaces in soil. Aromatic compounds get dissolved into water channels causing groundwater contamination. If sufficient amount of oxygen is present, then these can also be degraded biologically though gradually (U.S. EPA, 2006).

2.5 Sources of aromatic hydrocarbons in environment

Volatile organic compounds get into the environment through vehicular and air craft emissions, spills from different sources of petrol and losses during gasoline and other hydrocarbons marketing (Wang et al., 2010). Phenyl and BTEX components are being produced and consumed during the processing of coal, refined petroleum products and crude oil, during manufacturing of daily consumer products like adhesives, inks, cosmetic and pharmaceutical items. They can be introduced into the water through industrial wastewater and atmospheric emissions but discharge of aromatic compounds into water is mostly due to petrol spills (Nie et al., 2013).

2.6 Health effects of aromatic hydrocarbons

Volatile organic compounds can easily move into the atmosphere, so they can affect public health directly or indirectly. Short term exposure to these compounds show potential toxicity to marine life in water systems (especially in confined regions) as well as toxic breathing hazards. Chronic exposure of these chemicals causes alterations in liver functioning and life threatening effects on central nervous system, lungs and heart. When humans are exposed to such toxic compounds in the form of a mixture, it leads to genetic, neurological, excretory and respiratory system malfunctioning (Dastgheib et al., 2011). These chemicals are intense eye, skin and mucous membrane irritants and ultimately reduce bone marrow function and cause cancer. These chemicals

persist in the environment due to their wide spread applications in industrial processes (Tapilatu et al., 2010).

2.7 Degradation techniques

The conventional approaches for management of oil leakage and spills are being limited to physical methods. On the other hand, biological methods for treatment can have benefit over physico-chemical processes in removing spills as they present bioremediation of oil fraction by bacteria (Chaillan et al., 2004). Use Biological decontamination method, for oil and its derivatives in polluted surroundings is considered as a proficient, cost effective and resourceful alternate to other methods of waste treatment (Emtiazi et al., 2009).

Scope of bioremediation predominantly depends on oil concentration, length of alkanes, bio surfactants and also on the type of microbes used for degradation (Cappello and Richard, 2012). Based on observations, saturated components of crude oil are predominantly the alkanes of chain length (C10-C20) which can be easily degraded (Subarna and Raj, 2002). The level of uptake and mineralization of various organic complexes rely upon the amount of compound. High amount of hydrocarbons inhibit the process of degradation. It is done by either nutrient or oxygen shortage or via poisonous impacts applied by volatile organic compounds (Luis and Paul, 2000).

Iqbal et al. worked on the biodegradation of diesel by the use of bacterial strain named as P*seudomonas sp. J10* which has capacity to degrade the 69% diesel in just 4 days (Iqbal et al., 2019).

Das and Mukherjee (2007) stated petroleum hydrocarbons biodegradation by *Acinetobacter* species. Bacteria was capable of using n-alkanes as carbon source. Bacterial species like *Gordonia, Brevibacterium, Aeromicrobium, Dietzia, 3 Burkholderia* and *Mycobacterium* isolated from soil contaminated with petrol hydrocarbon are considered as potential degraders of hydrocarbons (Jain et al., 2010).

Another study stated biodegradation of polycyclic aromatic hydrocarbons by *Sphingomonas* species. Biodegradation of oil by synthetic microalgae-bacteria consortium was studied. Consortium that was made by *Scenedesmus obliquus* termed GH2 and other four crude oil degrading microorganisms with well-known degradation abilities, like *Sphingomonas* GY2B, *Burkholderia cepacia* GS3C, *Pandoraea pnomenusa* and *Pseudomonas* GP3A were said to be capable of eliminating alkanes totally, alkyl benzene and alkyl cycloalkanes within a period of seven and ten days, correspondingly. Consortium also favorably degraded polycyclic aromatic hydrocarbons like phenanthrene and methyl phenanthrene (Daugulis and McCracken, 2003).

2.7.1 Phytoremediation

Plant use solar power to extort, impound and detoxify the organic and inorganic contaminants from soil and water. It is termed as phytoremediation that is considered as advanced, environmentally friendly and cost effective treatment (Yadav and Yadav, 2017) over the physical and chemical treatment options. In this context, use of biological systems like phytoremediation seems an attractive option.

Plants are best known for the provision of food, fuel and fiber resources. Since last two decades, their role for elimination of contaminants from soil and water has been significantly investigated. Plant uptake the organic contaminants from soil and translocate them in shoot via roots. Their capability to tolerate, accumulate and degrade organic pollutants make them desirable to be used for the remediation of polluted sites (Ying et al., 2011).

Organic contaminant's uptake in different plant parts depends on contaminants concentration in soil and characteristic of chemicals (Lu et al., 2010). After the uptake of the contaminants, they can experience different pathways within plant body. Organic contaminants can be sequestered in roots, translocated to shoots and leaves, stored in the vacuoles or volatilize in the air (Ying et al., 2011). Biodegradation is considered as the main mechanism involved for the phytoremediation of hydrocarbons in rhizosphere (Vangronsveld et al., 2009). Plant species with extensive root systems and their tolerance to higher concentration of organic contaminants have been investigated with more emphasis on grasses (Lu et al., 2010). Grasses have been preferred because their extensive root systems have more surface area and deeper penetration in the soil. They can efficiently uptake and translocate higher concentrations of organic contaminants without any nutrient supplements (Hall et al., 2011).

Several plant species have been investigated for their efficiency in hydrocarbon removal from soil. *Sorghum biocolor* L. (sorghum), *Lolium multiflorum* (ryegrass), *Lotus corniclatus* (birdsfoot trefoil), *Zea mays* (maize), *Medicago sativa* (alfafa), *Cynodon dactylon* (bermuda grass) and several legumes are

reported to have more tolerance to higher concentration of hydrocarbons and are efficient in removal from soil (Hall et al., 2011; Yousaf et al., 2010; Tang et al., 2010).

2.7.2 Bioremediation

Use of microbes or microbial processes for the remediation of polluted sites or ground water is termed as bioremediation. Researchers have already reported the potential of aerobic as well as anaerobic biodegradation of the contaminants and transformation process that ultimately describes the outcome of the pollutant. Biodegradation of contaminants within root zone has already been reported many times (Karthikeyan and Kulakow, 2003; Zahid et al., 2016).

Bioremediation is the most preferred biological method for transforming and degrading harmful substance. This technique includes the biological apparatus using the regular cycles for remediation of tainted site. Toxic compounds were utilized by the microbes as a source of carbon and energy by breaking them down into smaller number of detrimental products (Azubuike et al., 2016). This methodology has been broadly used for remediation of hydrocarbon for terrestrial and aquatic ecosystem (Scoma et al., 2016).

Bioremediation is one of the "Top 10 Biotechnologies to Improve Global Health". Bacteria transform toxic compounds into less harmful substances in a process known as microbial bioremediation. Some examples are benzene, toluene, etc. Bioremediation in which micro-organisms for example, fungi or

bacteria are used to reduce hazardous compounds into less toxic substances is called microbial bioremediation (Pieper et al., 2000).

2.7.3 Phytoremediation of hydrocarbons

A multi-process technique was developed by Huang and coworkers to check the removal of PAHs from contaminated soil. This method composed of phytoremediation, photo-chemical, microbial and physical processes. This method was compared with the land farming, bioremediation and phytoremediation separately. Results shows that the degradation of 16 poly aromatic hydrocarbons in the multi process technique is twice than the land farming and 45% and 50% more than the phytoremediation and bioremediation respectively (Huang et al., 2004).

Teng and companions studied the effectiveness of the Alfafa plant association with the *Rhizobium meliltoi* for 90 days. Soil used for experimental purpose was agricultural soil, contaminated with polycyclic aromatic hydrocarbons (PAHs). Results of the study showed that the uninoculated alfafa plants decreased the concentration of the PAHs 37.2%, on the other hand inoculated plants lowered the contamination level by 51.4% compared with unplanted soil (Teng et al., 2011).

Jeelani and companions investigated the effect of polycyclic aromatic hydrocarbons (PAHs) and Cd on the plant *Acorus calamus* and its ability to accumulate the contaminants. Results show that biomass of *Acorus calamus*

increased by the addition of Cd and PAHs, so this plant can be used for the phytoremediation purposes (Jeelani et al., 2017).

2.7.4 Plant association with microbes for degradation

Microorganisms and Plant relationship for phytoremediation of polluted soil and water is a promising approach and is being investigated now a day. Increase in plant growth by bacteria can be due to nitrogen fixation process or production of phytohormones. Plants and Bacteria correlate to uptake, detoxify or eliminate the impurities from soil and water (Afzal et al., 2012; Baoune et al., 2019).

Bacteria compartmentalize in the plant body and produce various compounds that provide nutrients for plant growth or help plants to reduce organic contaminants stress. Improved plant growth or biomass production or hydrocarbon degrading bacteria effectively decrease organic contaminants in plant body (Baoune et al., 2018). Sharma et al. developed a consortium with *Pseudomonas putida* and *Bacillus subtilis* to check its effects on the growth of mung bean plant. Results of the experiment showed considerable increase in the seed germination by 18.73% (Sharma et al., 2018).

Saleem and coworkers applied bacterial consortium of the Acinetobacter, *Pseudomonas sp. and Bacillus cereus* to a wetland plant *Typha domingensis*. Findings showed that the plant was capable to degrade a very lesser amount of phenol from the polluted water but plants which were inoculated with consortium had more ability to accumulate and degrade phenol. The plant

biomass also increased, and a very high reduction in the level of COD, BOD and TOC achieved. Similar study was conducted with *Phragmites australis* with same set of bacteria and results were almost same. (Saleem et al., 2018; Saleem et al., 2019).

2.8 Aromatic hydrocarbons degradation

Bioremediation of petroleum products in the surroundings is difficult process. Quantitative and qualitative adjustments generally depend upon the nature and amount of petroleum product present, the ambient and intermittent ecological settings, such as dissolved oxygen, ideal temperature (20-35 °C) and physical or chemical dissemination of oil (Ubalua and Ezeronye, 2005).

Biodegradation of oil has been well-known to occur by attack of bacteria on light weight aromatics. High atomic weight aromatics like tars and asphaltenes are said to show amazingly low speeds of, though some studies conducted on bioremediation have stated their high removal rate under optimal conditions (Chang et al., 2013). The rate of degradation usually increases with increase in temperature such as ecological system exposed to low temperature can degrade aromatic compounds at a much slower rate. The degradation of petroleum hydrocarbons in aquatic ecosystem is restricted mainly by micro and macro nutrients like nitrogen and phosphorus. Salinity and pressure factors are essential in deep sea parts. Likewise dissolved oxygen, nutrient concentration, moisture and pH are major elements in the determination of degradation.

Petrol mixtures bind to soil particles and they are hard to be removed (Barathi and Vasudevan., 2001).

Petroleum hydrocarbons pollution in soil generally results in disproportion in carbon nitrogen fraction which ultimately causes nitrogen shortage in oil water logged soil, therefore hindering the growth of microorganisms and consumption of carbon sources (Bajpai et al., 2011). Moreover, high concentration of degradable compounds in the upper layer of soil diminishes oxygen in soil and slows down the degree of oxygen circulation. Many native bacteria in water and soil have ability to remove petroleum toxins. Petrol hydrocarbons can be biodegraded by various groups of bacteria, which are habitual of consuming aromatic compounds as nutrient for growth (Das and Chandran, 2011).

The biodegradation of complex compounds like crude oil and metals need bacterial consortium with extensive enzymatic capabilities (Bartha and Bossert, 1984). Brooijmans et al. studied the degree of degradation of non-volatile petrol compounds and the supplementary bacterial activity in major cumulative sizes throughout a pilot-scale bio pile trial carried out at temperature 15 degree Centigrade with soil having clayey texture, from a crude oil contaminated site in North of Canada. At the 65th day of the experiment, 42% of the C16-C34 hydrocarbons were found to be removed in the nutrient modified macro masses, parallel to 12% in the meso aggregates (Brooijmans et al., 2009).

2.9 Phenol

Phenols are the compounds which can be described as the derivatives of benzene. These derivatives of benzene are considered to be a great concern. For example, chlorophenols are the most harmful compound among this group and are in the priority pollutant list in US Environmental Protection Agency, because of its resistance to degradation, and its potential to harm the environment (Yamaga et al., 2010).

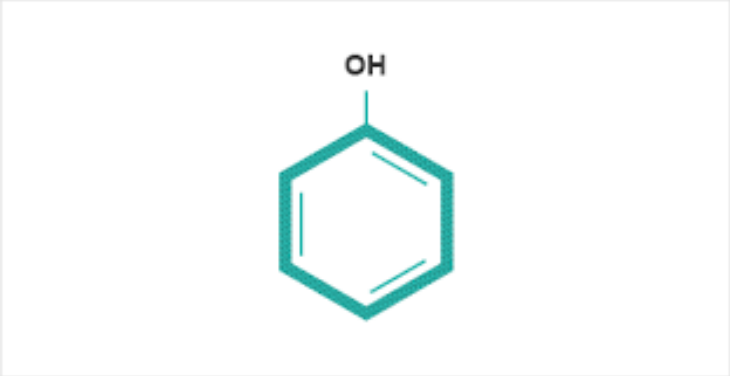


Figure 2.1: Phenol structure

2.9.1 Regulations on phenol

Phenols are aromatic compounds that are major toxins in effluent and wastewater from synthetic substances, petrochemicals, drugs, materials, and steel businesses (Rocha et al., 2007).

Table 2.1: Physical and chemical properties of phenol

Physical and chemical properties	
Name	Phenol
Formula	C_6H_6OH
Color	Colorless / white

State	Solid
Odor threshold	0.04 mg/L
Molecular weight	94.1 g/mol
Solubility	Soluble in water

The unwholesome and naturally prohibited contamination effects of the phenolic effluent have been represented the world over. Phenol contaminants are commonly dissolvable in water and gather in soil, achieving expansive surface water, ground water, and contaminating soil leads to severe harmful effects. Presently phenol effluents removal from contaminated sites has been a significant natural concern (Mahiudddin and Fakhruddin, 2011).

As per the U.S. Environmental Protection Agency (EPA), the most extreme permitted concentration of phenol in aquatic environments is 0.6 mg/L on a 24-h normal, failing to exceed 3.4 mg/L.

2.9.2 Degradation pathway of phenol

The common way to set up aromatic substances like phenol is to di hydroxylate the benzene ring and draw the catechol along the line to open the ring through ortho or meta oxidation. Catechol is oxidized by methodologies for ortho cleavage pathway by catechol 1,2-dioxygenase, or by meta pathway to 2-hydroxymuconic semi aldehyde by catechol 2,3-dioxygenase. The last postponed consequences of both the pathways are atoms that can enter the Krebs' cycle (Mahiudddin and Fakhruddin, 2011).

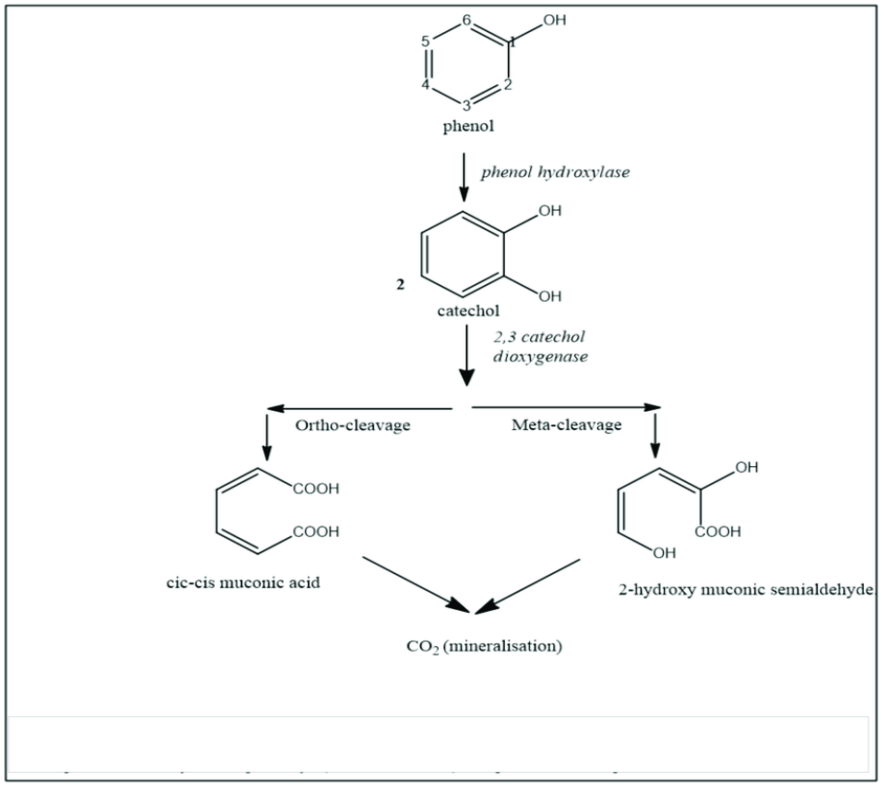


Figure 2.2: Phenol degradation pathway (Nzila, 2018)

2.10 Plants used for degradation of phenol

Plants are best known for provision of food, fuel and fiber resources. Since last two decades their use for the removal of contaminants from soil has extensively been studied. Their ability to tolerate and degrade the contaminants make them desirable to be used for the remediation of the contaminated sites (Ying et al., 2011). In 1992, Sandermann coined the term "green liver" for plants being responsible for metabolic processing of Xenobiotics. Hence plants act as "green liver" being natural sink for the contaminants (Sandemann., 1992).

In 2007, Singh and teammates worked on the evaluation of ability of *V. zizanoides* for the remediation of phenol. Phenol was discovered to be totally degraded from incubation medium toward the completion of 4 days by *V. zizanoides* plantlets, when medium was progressed with 50 and 100 mg/L phenol, while with 200, 500, and 1000 mg/L of phenol, 89%, 76% and 70%, freely, were taken out (Singh et al., 2008).

Oller and companions worked on the colonization of tomato hairy roots to check the degradation of phenol with all of the colons. This research examination came about that designed hairy root culture overexpressing tpx1 peroxidase upgrade phenol evacuation and could be valuable approach in phytoremediation process (Oller et al., 2005).

2.11 Role of consortium in degradation of phenol

Poi et al. developed consortium for treatment of phenol from the effluent (wastewater). Consortium used was the combination of *Bacillus sp., Acinetobacter sp.* and *Pseudomonas sp.* and developed a biofilm to degrade phenol. A study revealed that consortium was able to degrade the phenol below detection level as well as decreased the COD level of the wastewater (Poi et al., 2017). Ontañon and his coworkers conducted a study to check the efficiency of the consortium. This consortium was developed from *A. guillouiae Bacillus sp.* and *Pseudomonas sp.* Results of the study show that consortium helped to remove an increased level of Cr (VI) and also able to reduce up to 1500 mg/L of phenol from contaminated water (Ontañon et al., 2017).

Another study was conducted by Li and his coworkers to determine that weather bacterial consortium is efficient in high salinity conditions or not. A consortium of *Bacillus* and *Coryne bacterium* was developed. Concentration of phenol was 100 mg/L with 2% of NaCl. Bacterial consortium was able to achieve the results within 8 hours and degrade phenol below the detection level (Li et al., 2018).

To conclude this literature part, it is evident that there is significant potential in plants and microbes to degrade the phenol in the environment. The effectiveness and efficiency are dependent upon the right set of conditions and combination of plant and microbial species. An effort has been made in this study to develop an effective system through optical combinations.

Chapter 3

Materials and methods

Present study was performed in Environmental Biotechnology Laboratory, Institute of Environmental Sciences and Engineering at the National University of Sciences and Technology, Islamabad. Biodegradation studies of phenol were carried out by using bacterial consortium in four different bioreactors. Treatment methods in four of them were with plants (Ryegrass), bacterial consortium, plants inoculated with consortium and one was control. The increase in growth of bacteria determined the utilization potential of phenol by microorganisms and led to decrease in organic matter. To determine the extent of degradation quantitative analysis was done by UV- visible spectrophotometer to correlate the reduction in phenol content with the corresponding growth of microbes and used plant biomass.

3.1 Chemicals and standards

Chemicals used in this research work are stated below.

Table 3.1: List of chemicals used in this study

Chemical	Manufacturers	Purpose
Ethanol (C_2H_5OH)	BDH AnalaR, England	Soxhlet extraction of plants
Dipotassium phosphate (K_2HPO_4)	Sigma-Aldrich, USA	Potassium buffer

Monopotassium phosphate (KH_2PO_4)	Sigma-Aldrich, USA	Same as above
Ammonia solution (NH_4OH)	Sigma-Aldrich, USA	Ammonia buffer
Sodium carbonate (Na_2CO_3)	Merck KG AA, Germany	Plant uptake analysis
Ammonium chloride (NH_4Cl)	Sigma-Aldrich, USA	Mineral salt media
Sodium chloride(NaCl)	Merck KG AA, Germany	Same as above
Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$)	Aldrich-Chemie, Germany	Same as above
Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$)	Sigma-Aldrich, USA	Same as above
Calcium hypochlorite ($Ca(ClO)_2$)	BDH AnalaR, England	Seed sterilization
4-Aminoantipyrine $C_{11}H_{13}N_3O$	Merck KG AA, Germany	Phenol detection
Folin–Ciocâlteu reagent ($C_{10}H_5NaO_5S$)	Sigma-Aldrich, USA	Phenol concentration in plants
Dimethyl sulfoxide (C_2H_6OS)	Sigma-Aldrich, USA	Chlorophyll analysis

Potassium ferrocyanide ($C_6FeK_4N_6$)	Merck KG AA, Germany	Phenol detection

3.2 Selection of plants and bacteria

Plant which was selected for the experiment was ryegrass (*Lolium perenne*) to be tested in combination with consortium of selected bacterial strains. According to recent studies, *L. perenne* has been recommended as a plant which may withstand higher hydrocarbon (Iqbal et al., 2019) concentration because of its wide root system, fast growth and ceaseless nature (Ruiqin et al., 2013). Selected microbial strains were *P. putida* and *P. aeruginosa* due to their ability to degrade the hydrocarbon (Iqbal et al., 2017).

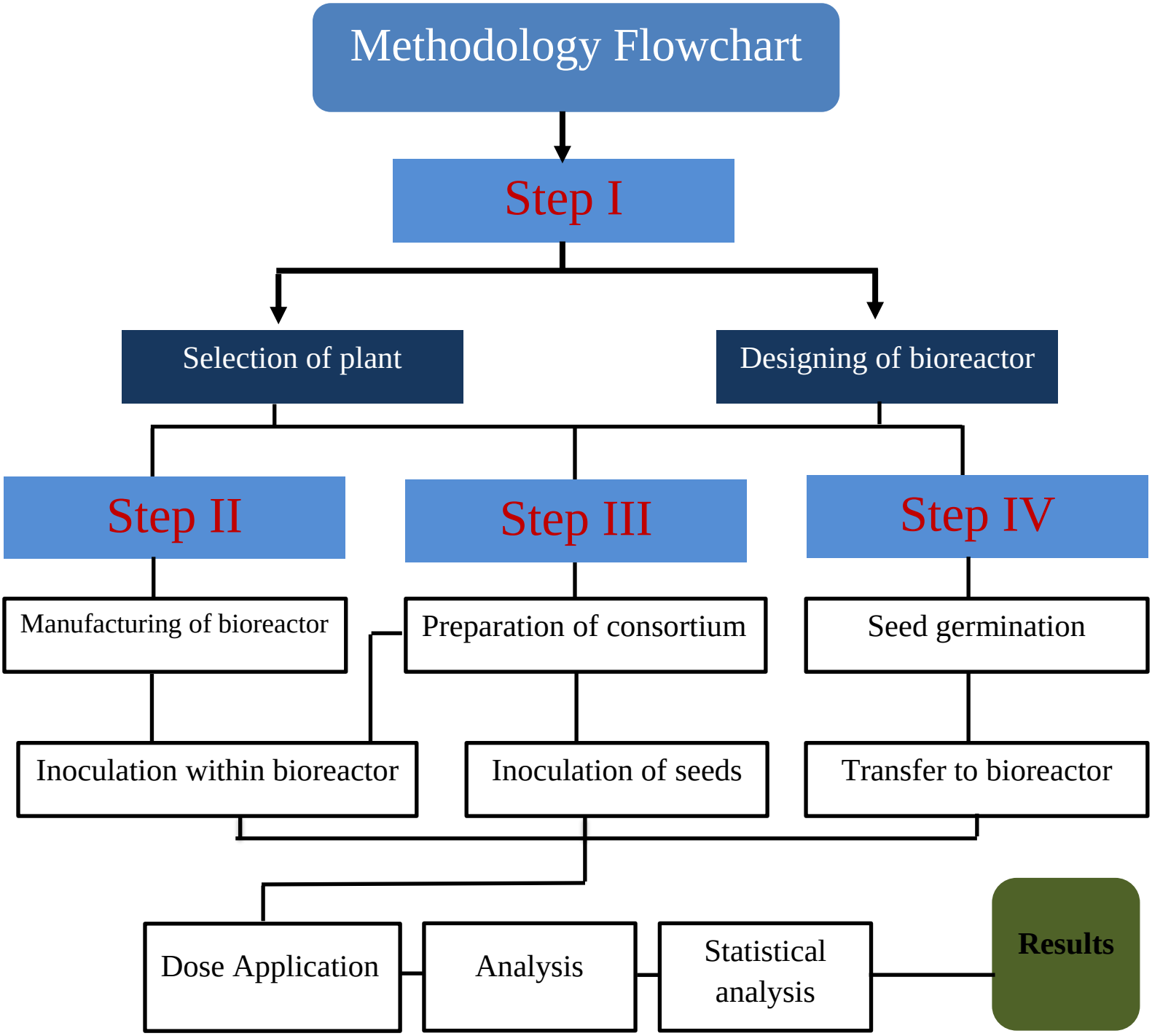
3.3 Glassware washing and sterilization

Glassware used in this experiment was washed using tap water, afterwards distilled water was used for rinsing before experiment. All the glassware was autoclaved at 121 °C, 15 psi pressure for 15 min for sterilization. Sterilized glassware was oven dried at 105 °C for 2 h. All glassware for spectrophotometric analysis was assembled prior to sampling through washing with phosphate free detergent then with chromic acid and final rinsing with distilled water.

3.4 Preparation of soil

Soil from the nursery of National University of Sciences and Technology, Islamabad, Pakistan was selected for the final experimentation. Soil was firstly

spread out and air dried for 48 hours. The dried soil was grounded into fine form by using pestle and mortal. In last preparatory stage, soil was sieved using a 2mm sieve prior to use for the experimentation.


Methodology Flowchart
Step I
Selection of plant
Designing of bioreactor
Step II
Step III
Step IV
Manufacturing of bioreactor
Preparation of consortium
Seed germination
Inoculation within bioreactor
Inoculation of seeds
Transfer to bioreactor
Dose Application
Analysis
Statistical analysis
Results

3.5 Experimental setup

For the experiment, four reactors were used. Each reactor was 32 inches long and 8 inches wide. The reactor consists of four parts, the lowest part was filled with pebbles up to 5 inches' height, then 7 inches layer of gravel, after that 7 inches' layer of sand and upper most layer was of soil which was also of 7 inches. Plants were grown in the top most soil layer as shown in Figure 3.1.

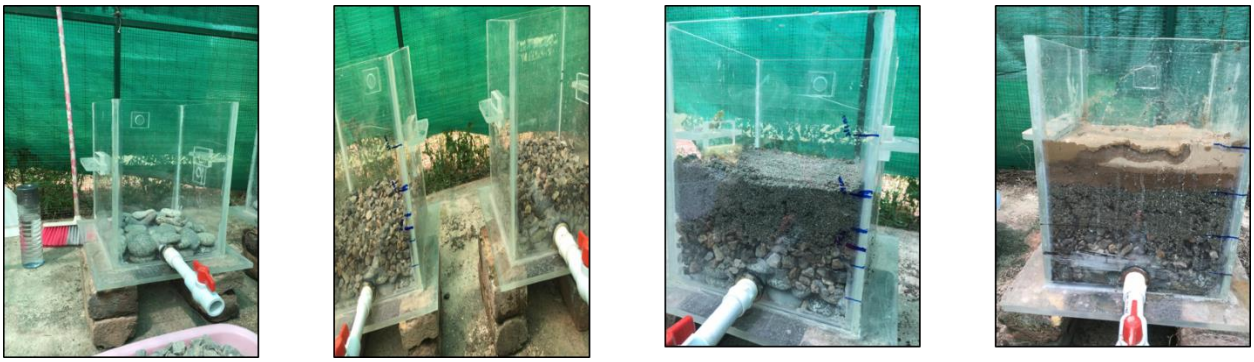


Figure 3.1: Pictorial view of bioreactor setup

3.6 Preparation of media

3.6.1 Mineral Salt Medium (MSM)

The composition of mineral salt medium is shown in table 3.1.

Table 3.1: Composition of mineral salt media

Composition of mineral salt medium		
Sr. no	**Chemicals**	**Quantity (g/L)**
1	K_2HPO_4	1.8
2	NH_4Cl	4.0
3	NaCl	0.1
4	$MgSO_4.7H_2O$	0.2
5	$FeSO_4.7H_2O$	0.01

3.6.2 Nutrient agar

Merck KGA nutrient agar was used for the experimental studies. The media was prepared according to the manufacturer ‘s instructions. Conical flasks containing nutrient agar were used to make nutrient agar plates. Conical flasks were autoclaved at 121 °C, 15 psi pressure for 15 minutes. Nutrient agar medium plates were made in sterile conditions using Lamina flow hood chamber and incubated for 24 h at 37 °C. Prepared plates were used for microbial isolation, purification, inoculum preparations and degradation studies (Ramanathan and Lalithakumari, 1999).

3.6.3 Nutrient broth

To prepare fresh cultures and bacterial inoculum, nutrient broth was preferred as per instructions provided by the manufacturer. For this purpose, 14 g of nutrient broth (Merck KGA) was mixed in 1 L distilled water and autoclaved at 121 °C and 15 lb/cm² pressure for 15 minutes. Sterility test was performed by placing conical flask of nutrient broth in incubator at 37 °C for 24 hours (Kao et al., 2005).

3.6.4 Maintenance of bacterial culture

Isolated microbial strains were streaked onto nutrient agar plates and microbial growth was observed after incubation at 37^{o}C for 24 hours and cultures were kept at 4^{o}C to make it useful for longer period of time. Bacterial cultures were sub-cultured after every two weeks for the experimental studies**.**

3.7 Bacterial consortium preparation

3.7.1 Growth of bacteria

For growth of bacterial isolates, broth cultures were prepared by using the same media except agar. Both the bacterial strains were streaked on petri plates of agar before 24 hours. Developed colonies were picked with the help of inoculating needle and inserted in conical flask having 500mL of broth. Inoculated flasks were than incubated for 48 hours at 30^{o}C using rotary shaking incubator at 120 rpm (Ho et al., 2012).

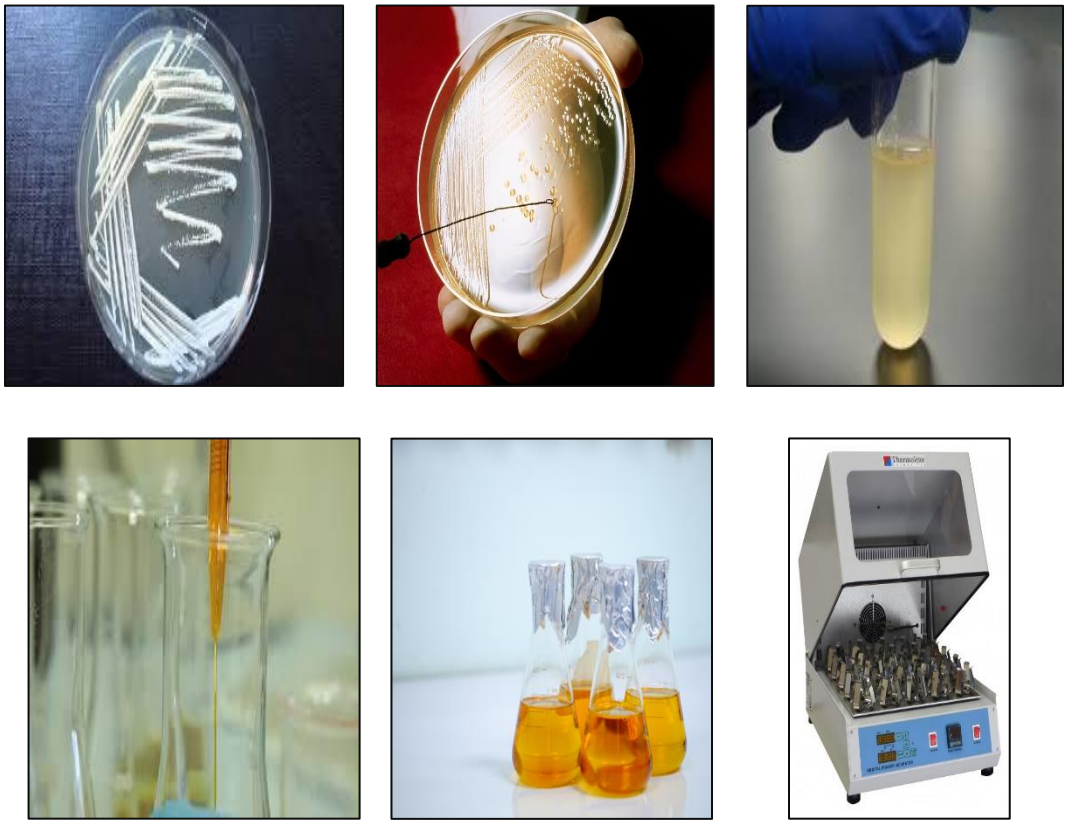


Figure 3.2: Bacterial consortium preparation

3.7.2 Optical density

Bacterial strains growth was determined by measuring optical density (OD) of different bacterial strains at 600 nm, using spectrophotometer before starting batch experiment. In order to calibrate the instrument before using, 10 mL distilled water was used. The OD of the broth was expected to 3.

3.7.3 Consortium preparation

After measuring OD, broth was transferred to autoclaved falcon tubes and centrifuged at 6000 rpm for 10 minutes. The centrifuged bacterial strains were mixed with autoclaved MSM media and checked its OD again. Then diluted the solution up to 10 folds (Ho et al., 2012).

3.8 Plant preparation

3.8.1 Seed surface sterilization

Ryegrass *(L. perenne)* was selected as test plant species to analyze the phenol degradation capacity. Healthy seeds of ryegrass were selected and sterilized in 5% solution of calcium hypochlorite for 5 minutes. Seeds were washed thrice with ultrapure autoclaved water before final utilization.

3.8.2 Seed germination

Petri dishes were washed and left to be air dried at room temperature. Filter papers were placed in these dishes and 15 seeds were placed in each Petri dish. Filter paper was then moistened with different treatment methods i.e., seeds with consortium, seeds with germination solution and control with distilled water.

These Petri dishes were then placed in an incubator (Stuart orbital incubator S150, UK) at 18°C for germination for 7 days.

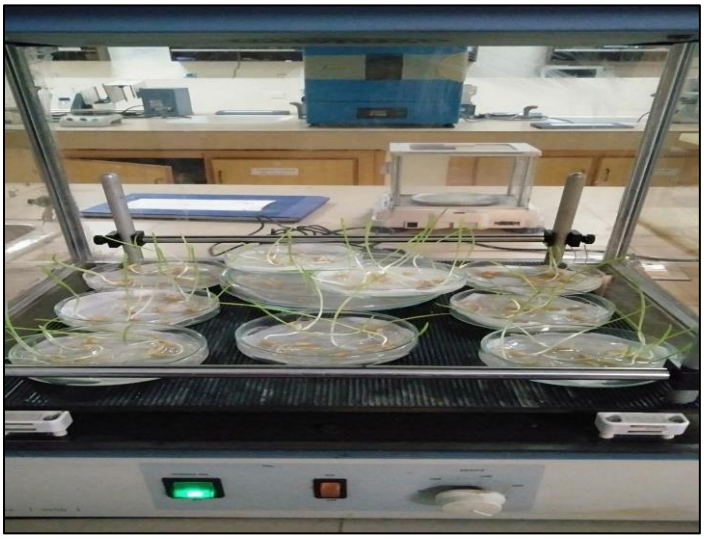


Figure 3.3: Seed germination setup

3.8.3 Germination test

After seven days of incubation, seedling germination rate and length of seedlings were measured from all the Petri plates using simple measuring scale.

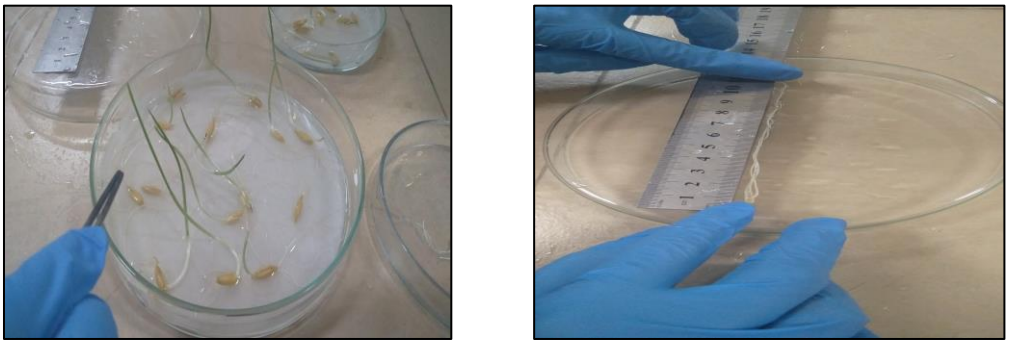


Figure 3.4: Seed germination test

3.8.4 Plant cultivation

Seeds were germinated in the petri plates. After 7 days, seedlings were shifted into the bioreactor and acclimatized for further seven days. After the acclimatization process 250 mg/L of phenol was applied to check the efficiency of control plants and plants inoculated with bacterial consortium. Water samples were taken on daily basis for analysis of phenol concentration.

3.9 Concentration of phenol

For experimental studies, concentration of phenol was 250 mg/L was used for degradation studies in all four of the bioreactors in present research work.

3.9.1 Composition of aqueous solution

To prepare the synthetic wastewater, 250 mg of solid phenol was mixed with 1L of distilled water.

3.10 Analytical procedures

3.10.1 Water analysis

For the detection of concentration of phenol in the water samples, four reagents were used in 100mL of water, 2mL of ammonia buffer, phosphate buffer to set the pH of water up to 8, then 1mL of 4 amino anti-pyrine solution and at lastly 1mL of potassium fericynide solution which changes the color of sample into orange or red according to the concentration of phenol in water were added. Lastly, read absorbance at 500nm wavelength on UV visible spectrophotometer.

Figure 3.5: Phenol Analysis by 4AAP method

3.10.2 Chlorophyll analysis

100 mg of fresh grass were taken and placed in vial with 7 mL DMSO. Then vial was placed in the incubator at 65^{o}C for two hours. When the chlorophyll got extracted in the DMSO completely then the solution was shifted into a measuring cylinder and made up to 10mL with DMSO. A 3ml of solution was taken in cuvette and measured on a spectrophotometry at 645 and 663 against blank DMSO or acetone. If the value of the solution is more than 0.7, then chlorophyll solution was diluted up to 50% with 90% of acetone. Then the values put in the eq. I, II and III (Hiscox and Israelst,1979).

Chlorophyll a(µg/L)= (12.7*OD at 663nm)-(2.69*OD at 645) .....(I)

Chlorophyll b(µg/L)= (22.9*OD at 645nm)-(4.68*OD at 645) .....(II)

Total Chlorophyll(µg/L)=(20.2*OD at 645nm)-(8.02*OD at 645).....(III)

3.11 Phenol uptake in plants

3.11.1 Extraction of plant

The extraction of plant was done by soxhelt extraction method. Plant samples were collected, cleaned and dried at room temperature. After drying plants were crushed into powder form. After that samples were extracted by soxhelt extraction with 70% ethanol as solvent. Collected extract of plant was dried using rotary evaporator (Sembiring, 2018).

Figure 3.6: Plant extraction for phenol uptake analysis

3.11.2 Determination of phenol content in plants

Determination of phenol was done by Folin-Ciocalteau method. 1 mL of extract was mixed with 2.5 mL of Folin-Ciocalteau reagent. After 5 mins, 2 mL of Na_2CO_3 (75%) was added and placed at 50^oC for 10 mins. Then mixture was allowed to cool down and checked the absorbance at 765 nm on a UV visible Spectrophotometer. (Aryal et al., 2019)

3.12 Statistical analysis

Statistical analysis was performed on obtained results by applying one-way ANOVA using STATISTICA software. If probability of obtained results were less than 0.05 ($p < 0.05$), results were considered statistically significant.

Chapter 4

Results and discussion

4.1 Physico-chemical properties of soil

Physico-chemical properties of soil samples were determined by measuring soil pH (6.9) and Electrical conductivity 1.5 dS/m. Soil texture of selected soil was sandy loam, which makes it suitable for the growth of plants.

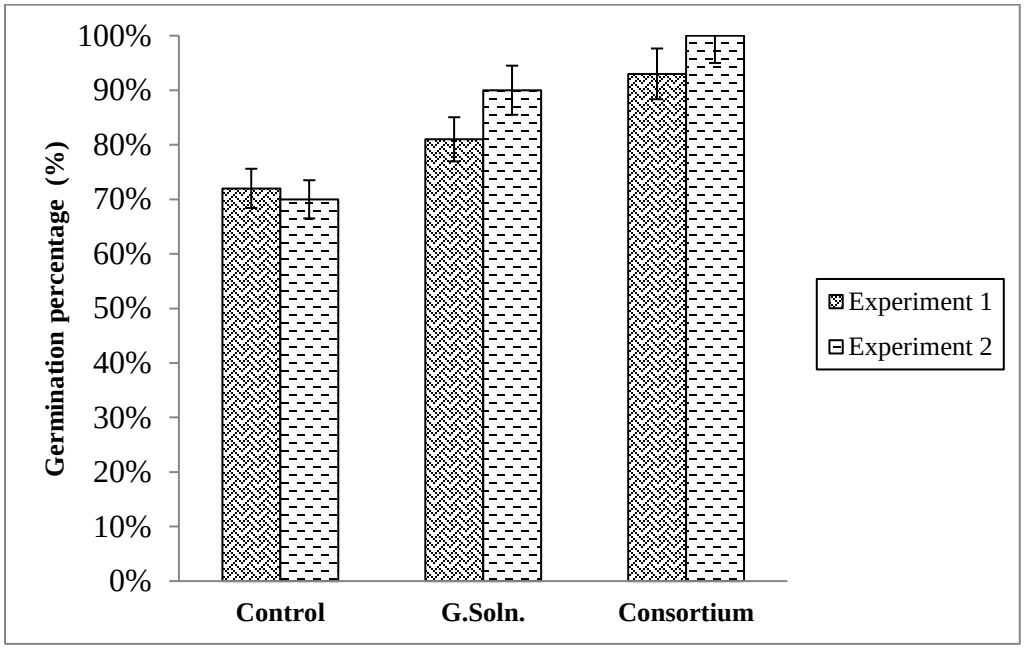
4.2 Bacterial strains

In order to monitor the ability of microbes to degrade phenol, two visually distinct bacterial strains were selected from *Pseudomonas genus*. These bacterial species were thought to be capable to use petroleum hydrocarbons as sole carbon source (Iqbal et al., 2017). However, enzyme systems and nutritional capabilities are also important factors which make the petroleum degraders to survive in adverse environmental conditions.

4.3 Seed germination

To observe the effects of bacterial consortium on seed germination, seeds of ryegrass (*L. perenne*) were exposed to the consortium (*P. putida* and *P. aeruginosa)* and germination solution and control was treated with distilled water. The results show that bacterial consortium has positive effect on the germination of seeds. Two sets of seeds with consortium had 93% and 100%,

germination rate, while seeds treated with germination solution and distilled water had less germination rate. The results are illustrated in the figure 4.1 depicting the effect of bacterial consortium, germination solution and distilled water on seed germination of ryegrass after seven days. These results conclude that seed germination is faster in the presence of bacterial consortium.



G. Soln. stands for germination solution

Figure 4.1: Seedling Germination percentage of Ryegrass.

4.4 Seedling length

Seedling length also increased in the presence of bacterial consortium. Results for the seedling length are mentioned in the below figure 4.2.

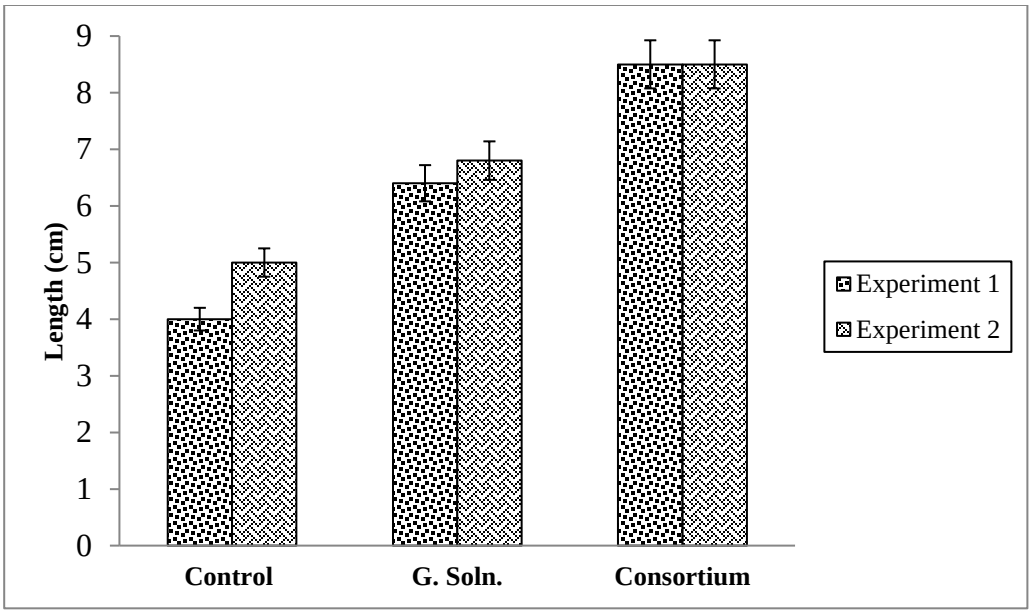


Figure 4.2: Seeding length of Ryegrass.

Hussain et al. (2018) reported that biomass of Ryegrass with consortium was 46% more as compared to control. Same study revealed that the height of inoculated plants with consortium is 79% more as compared to control (Hussain et al., 2018). Higher plant dry mass was observed in plant inoculated with microbial consortium than control plants (Mora et al., 2017). Consortium of *B. subtilis* and *P. aeruginosa* was applied in the pea plants to check its effects on the growth of plants and resistance to fungus. Fresh shoot mass was 1.83 folds higher than the control plants and consortium also proved helpful to improve resistance of plants against disease (Jain et al., 2015).

4.5 Biodegradation of phenol

Residual concentration of phenol was determined with UV spectrophotometer in order to estimate the biodegradation of phenol. It was confirmed that decrease in the concentration of phenol is the result of biodegradation, because controlled bioreactor showed no consistent reduction in the concentration of phenol. Comparison of profiles of different bioreactors to degrade phenol showed that the degradation occurred the slowest in controlled bioreactor.

For the biodegradation of 250 mg/L of phenol, four bioreactors were established with different treatment methods. Every treatment method had different rate of degradation at different time span. With all of the treatment methods, almost 90% of degradation achieved in 8 days. Degradation of phenol in all of the bioreactors with respect to days is shown in figures 4.3-4.10.

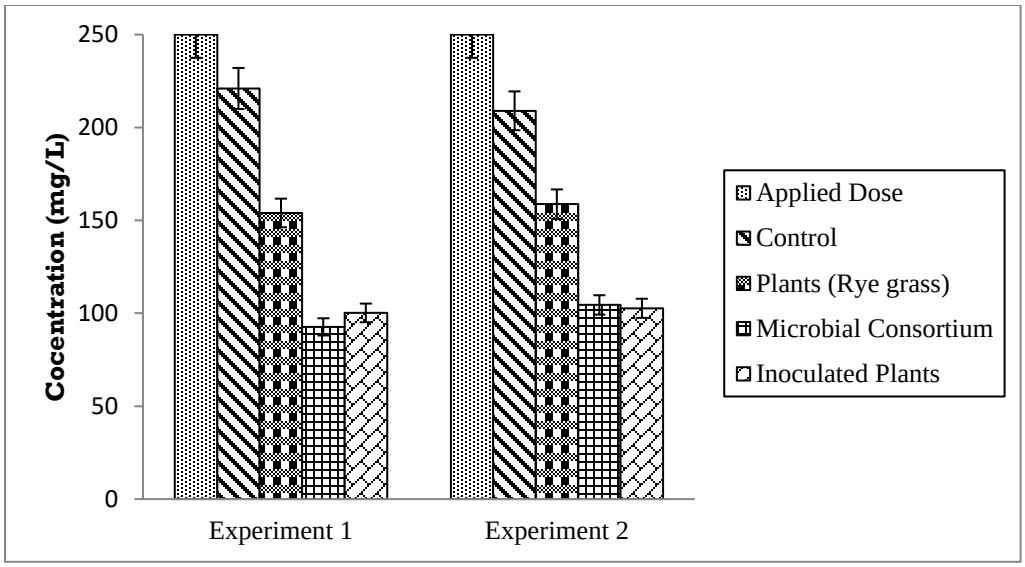


Figure 4.3: Residual phenol concentration after day one.

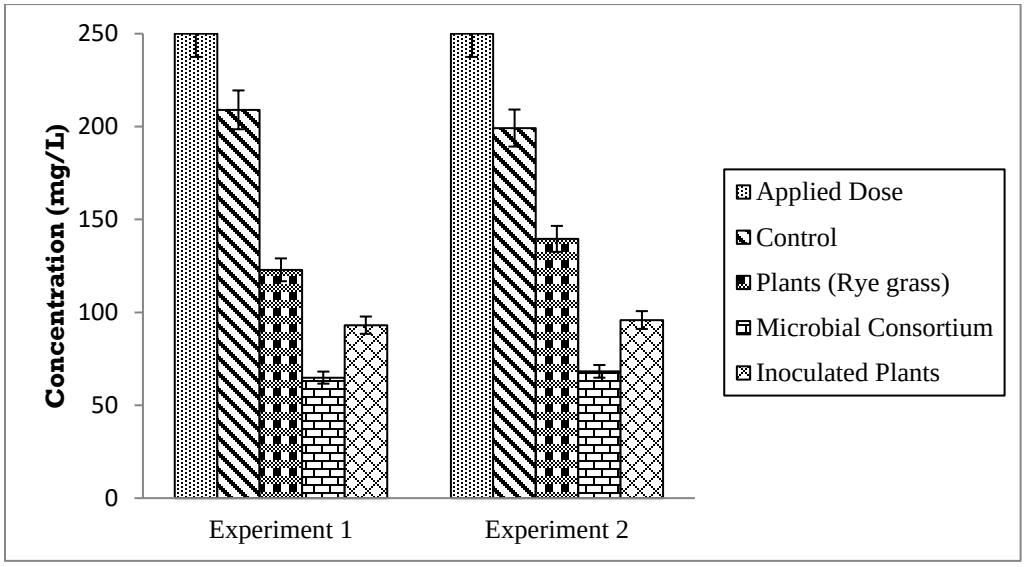


Figure 4.4: Residual phenol concentration after day two.

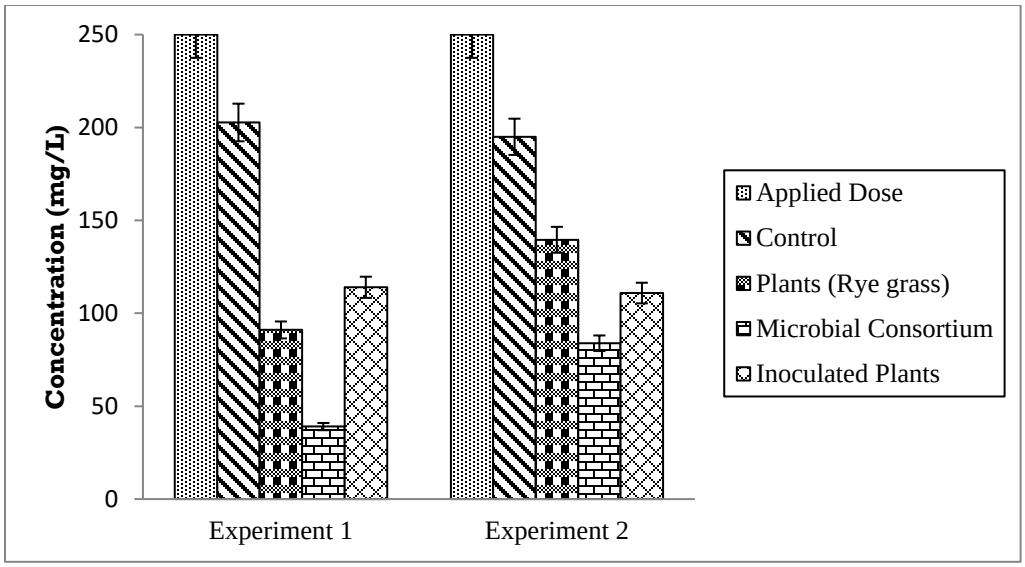


Figure 4.5: Residual phenol concentration after day three.

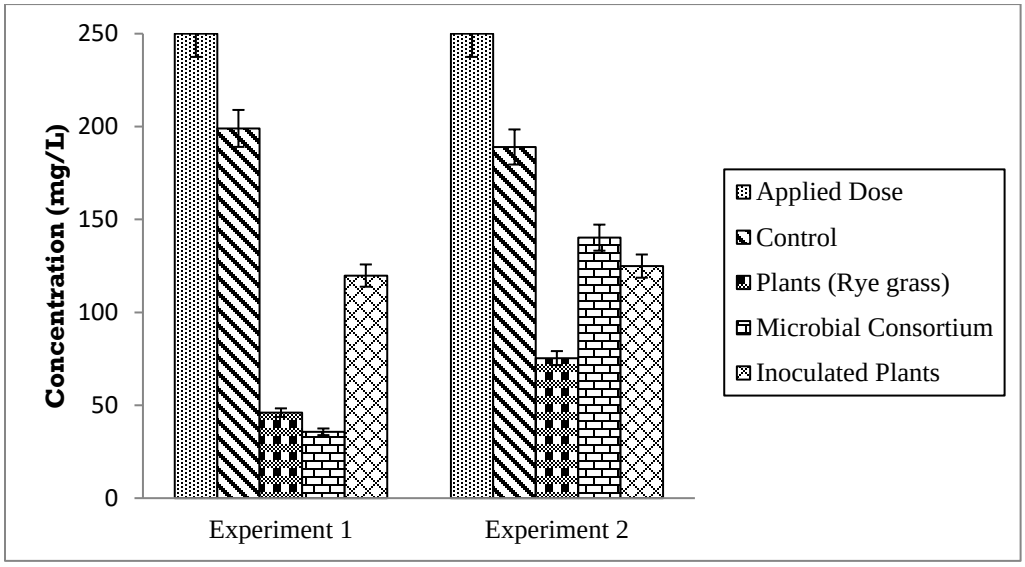


Figure 4.6: Residual phenol after day four.

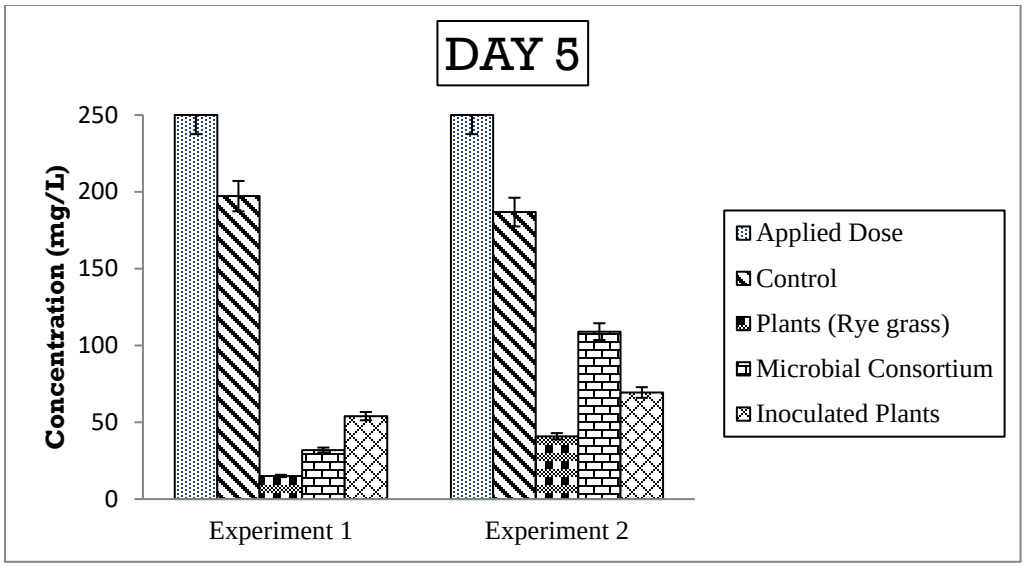


Figure 4.7: Residual phenol concentration after day five.

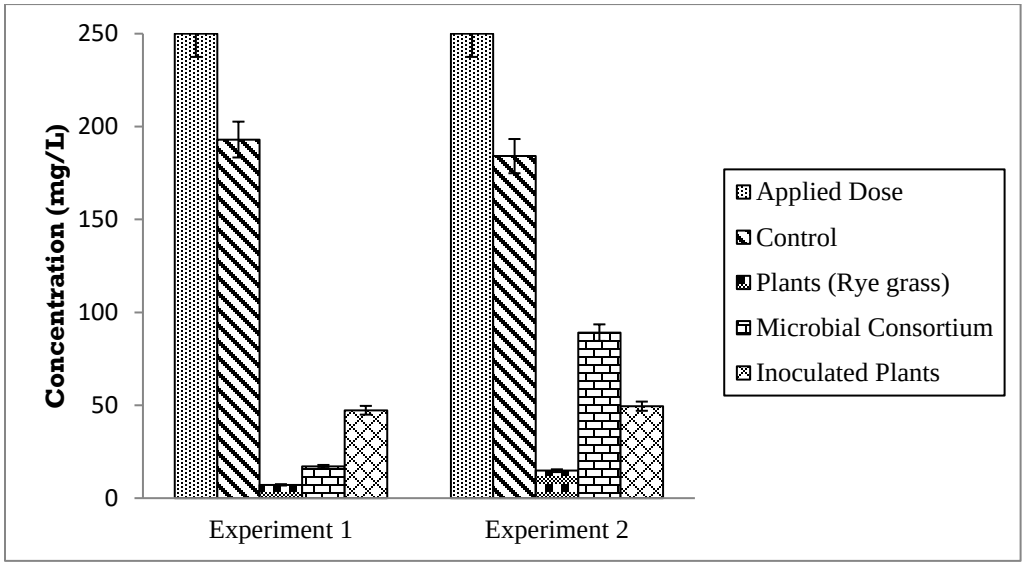


Figure 4.8: Residual phenol concentration after day six.

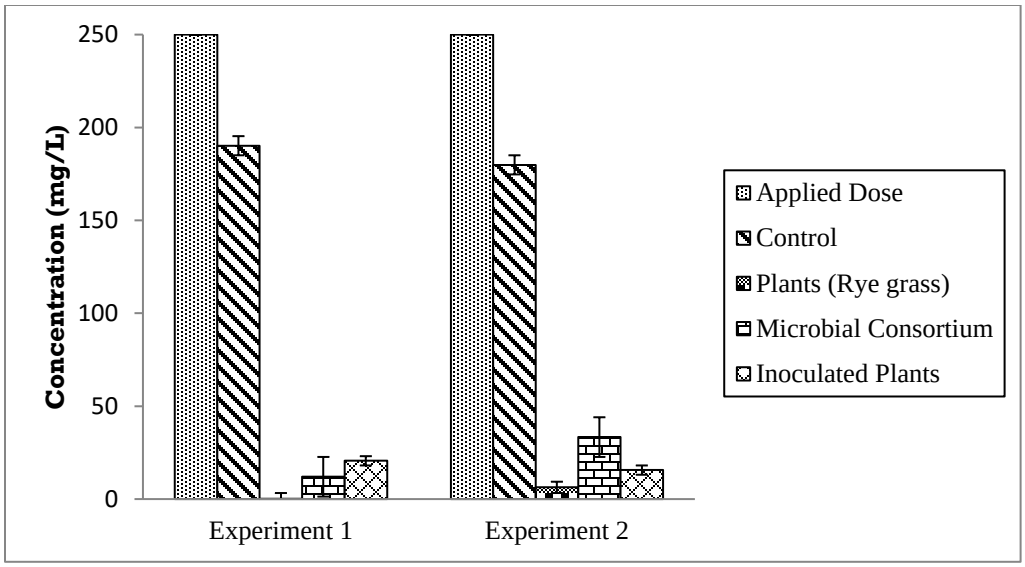


Figure 4.9: Residual phenol concentration after day seven

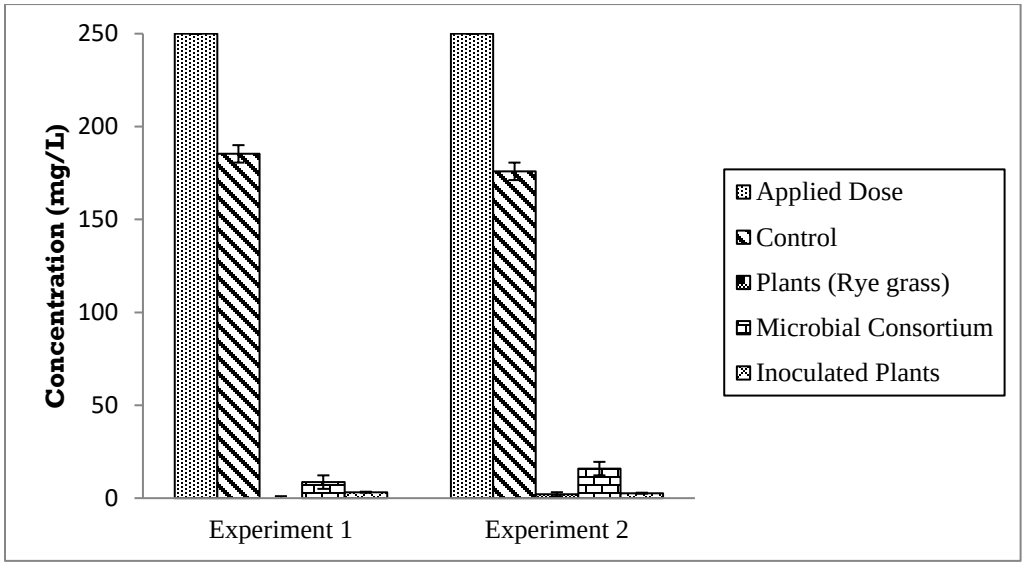


Figure 4.10: Residual phenol concentration after day eight.

Bioremediation is a promising technique that uses microorganisms and plants to treat contaminated sites. However, all the techniques have its pros and cons, sometimes plants can uptake toxic compounds from the environment but are not able to degrade efficiently and store contaminants in roots and shoots. When

plants die, these contaminants would reinstate in environment again. So to cope with this problem, different microbes are introduced in the plants to increase the capacity of plants to degrade the toxic contaminants.

Poi and coworkers reported that a combined consortium developed from different microbial strains was capable to degrade phenol to below detection level (Poi et al., 2017). A consortium of *Bacillus sp.* and *A. guillouiae* was applied in synthetic wastewater containing 25 mg/L of Cr and 300 mg/L of phenol. A complete removal of phenol was obtained in three days (Ontañon et al., 2017). Freshwater and sea water was mixed with 500 mg/L of diesel to make synthetic wastewater. After that, contaminated water was treated with the consortium of microbial strains *G. alkanivorans* and *R. erythropolis*. Freshwater showed 94% of decrease within 11 days of applying while sea water 85% removal of diesel (Chen et al., 2017).

Ryegrass was grown in soil polluted with chlorpyrifos, grass was able to uptake contaminant from soil but there was a decrease in the plant growth as compared to the plants grown lacking chlorpyrifos. *Bacillus pumilus* was inoculated in the plants to increase its efficiency, results show that inoculated plants had more biomass in the existence of 25 mg/kg and 50 mg/kg of chlorpyrifos in soil (Ahmad et al., 2012).

The ryegrass plants alone achieved a 22.2% rise in the fluoranthene cleaning level than soil without any treatment. The immunization of the soil with *B. cereus* and *A. pascens* inoculated in ryegrass further improved fluoranthene

elimination from the soil, with the removal amount of 35.6% and 37.7%, while the mixture of the two strains and ryegrass achieved the most fluoranthene decrease, which was 43.8% (Li et al., 2020).

4.6 Plant analysis

4.6.1 Phenol uptake

For the plant analysis soxhlet extraction was used at Institute of Environmental Sciences and Engineering. For this purpose, conditions were optimized for plants. Standard procedure (Sembiring, 2018) was used with some modifications after many trials, as described in materials and methods.

Determination of phenol in the extract was done by Folin-Ciocalteau method, extract was run on UV spectrophotometer at 765nm (Aryal et al., 2019) and the obtained results are mentioned in figure 4.11.

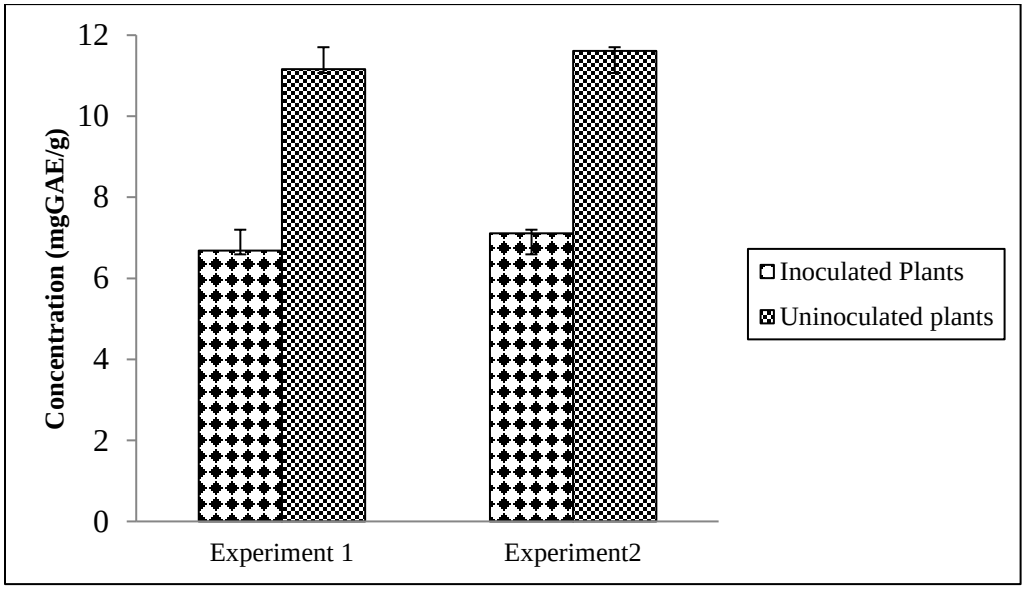


Figure 4.11: Phenol uptake in plants

These results show that inoculated plants store less phenol as compared to the control plants. This could be due to the presence of microbes which use the phenol as a sole carbon and leave a positive effect on the plants.

The competition between roots and microorganism for nutrients may inhibit the improvement of roots. Moreover, when they are used exclusively as seed inoculant as they have the ability to degrade common pollutants, microorganisms may give supportive effects upon plant advancement and along these lines improve the phytoremediation potential (Bokern et al., 1998). Degradation of phenantherene was faster in alfafa plants inoculated with inoculum (Harvey et al., 2002). Bacterial seed inoculants further clearly renew the ability of the rhizospheric community to degrade certain pollutants without disturbing heterotrophic microbial communities. The microbial community associated with plant roots has been used as a reasonable source of halogenated aromatic hydrocarbon degradation, for example, chlorophenol and nitro phenols (Caldeira et al., 1999).

4.6.2 Chlorophyll analysis

Chlorophyll analysis was done by DMSO method, chlorophyll a, b and total chlorophyll were measured by using the obtained value from UV spectrophotometry in equation mentioned in materials and methods section. Chlorophyll a is the type of pigment which is involved in the photosynthesis of plant while chlorophyll b is the accessory pigment which pass the energy to the chlorophyll a. Total chlorophyll is the sum of both “a and b”. Here only total chlorophyll is mentioned (Figure 4.12).

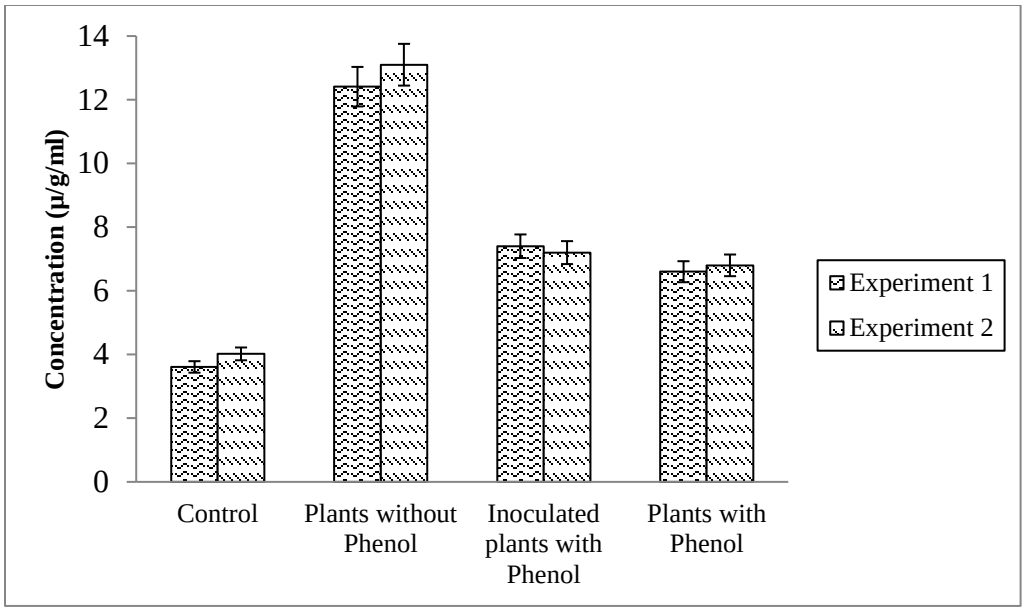


Figure 4.12: Total chlorophyll content

Results show that maximum chlorophyll was present in the plant which were not exposed to phenol. Plants which were germinated with germination solution and exposed with phenol have more chlorophyll as compared to plants which were germinated with distilled water. Inoculated plants were having a very less amount of increase in chlorophyll when exposed with phenol.

Chapter 5

Conclusions and recommendations

5.1 Conclusions

Based upon the experimental results and statistical analysis following conclusions can be drawn from the present study

1. The control setup had a low removal efficiency, whereby, 77 mg/L of the initially applied phenol dose was degraded.
2. The ryegrass showed an effective removal efficiency of 97%.
3. Plant inoculated with consortium yielded the highest phenol removal efficiency of 99%.
4. Plants and microbial consortium were able to degrade 250 mg/L phenol within 8 days.
5. Microbial consortium enhanced the efficiency of plants to degrade the pollutant, and also help to increase in the biomass of the plants.
6. Inoculated plants stored less phenol in the plant and most of the phenol was used by microbial consortium as a sole carbon.

5.2 Recommendations

Following recommendations are suggested:

1. The consortium of different strains could be applied in the field for the treatment of petroleum industry effluents having pollution load of hydrocarbons.
2. Consortium bacterial strains from different genera may be studied in combination for effective remediation of contaminated soils and water with phenol and other pollutants of similar nature.
3. Identification of genes responsible for phenol degradation in these bacterial strains could help to exploit their maximum potential for degradation through modification of relevant gene expression.

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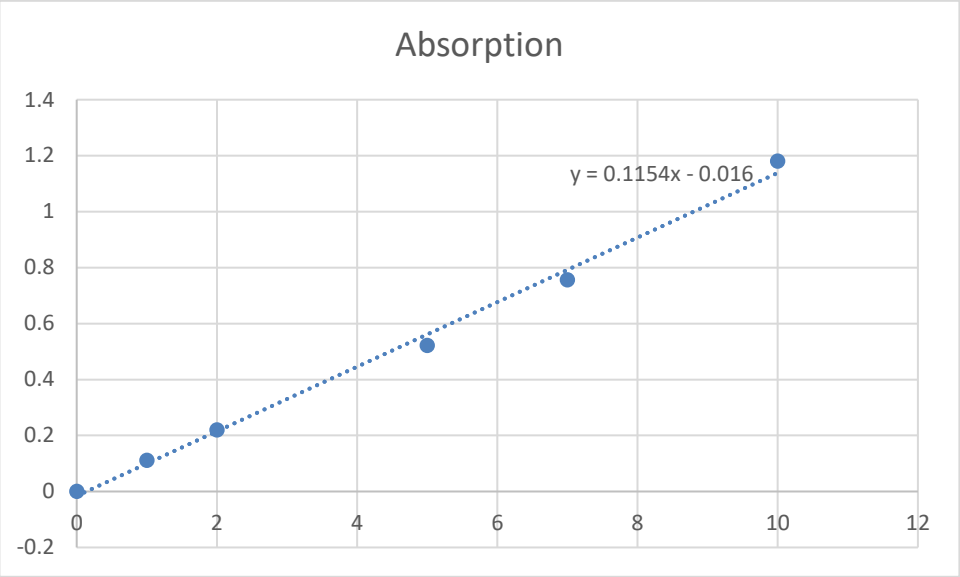
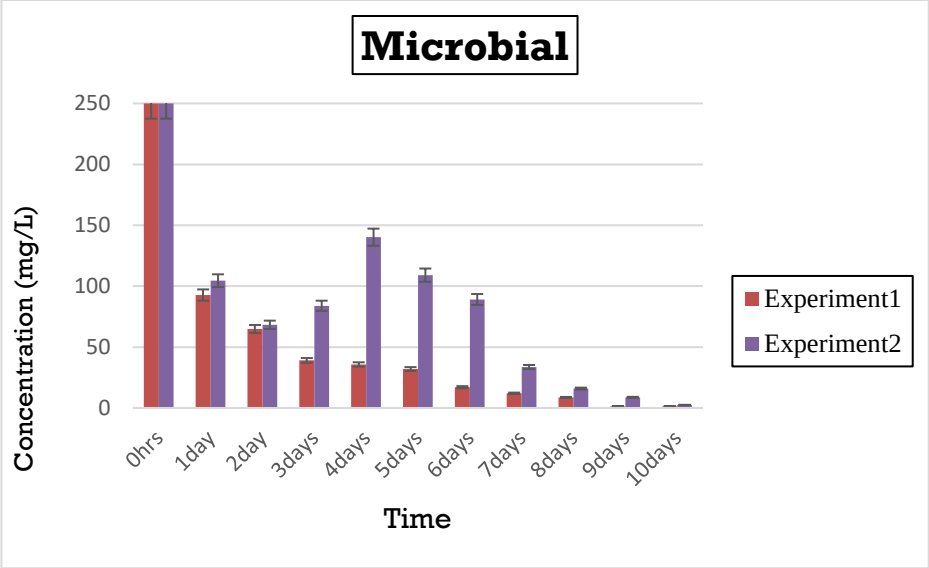
Annexure

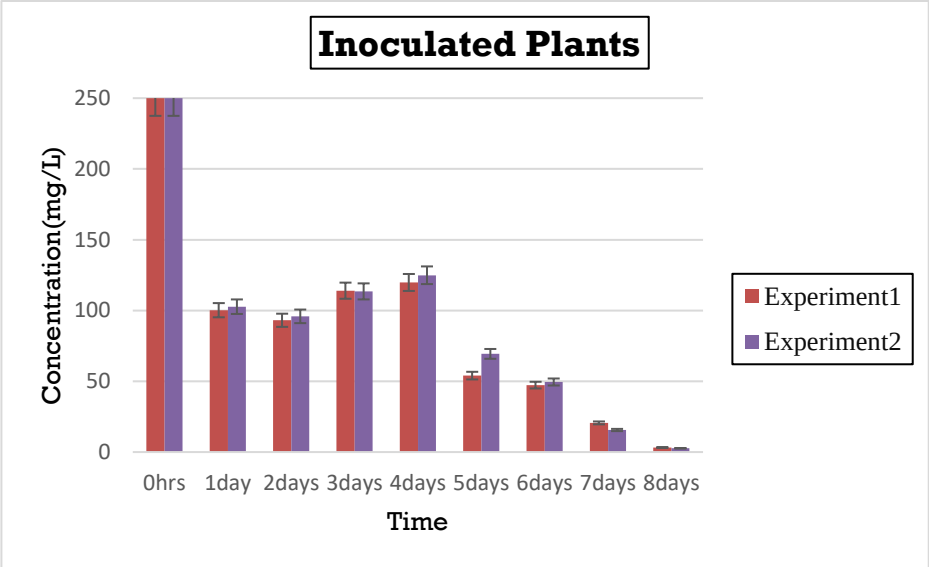


Phenol calibration

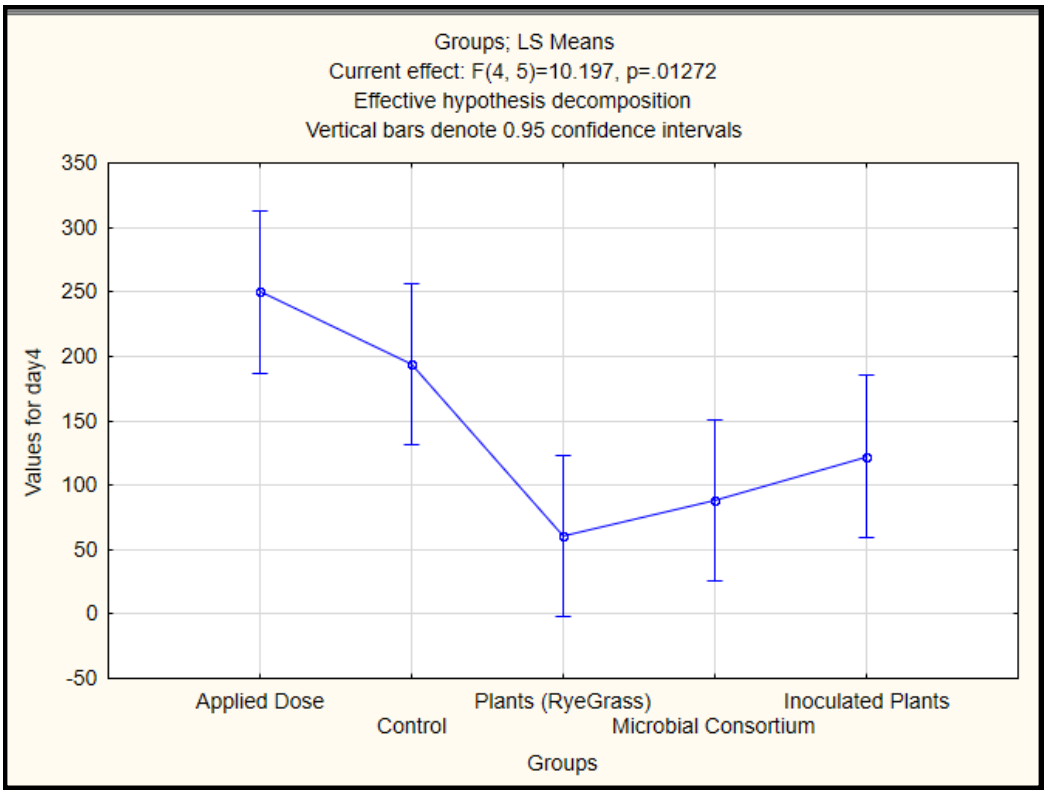
Plant uptake of phenol



Microbial uptake of plants



Inoculated plant uptake of Phenol



Statically analysis