Isolation and characterization of phenol degrading organisms from soil sample containing traces of crude oil

Thesis submitted by

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In partial fulfillment for the award of the degree of

Bachelor of Technology in Chemical Engineering

Under the guidance of

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2015

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CERTIFICATE

This is to certify that the thesis entitled **"ISOLATION AND CHARACTERIZATION OF PHENOL DEGRADING MICROORGANISMS FROM SOIL SAMPLE CONTAINING TRACES OF CRUDE OIL"** submitted by Ms. Megha Saluja in partial fulfillment of the requirements for the grant of Bachelor of Technology Degree in Chemical Engineering at the National Institute of Technology, Rourkela is an authentic work carried out by her under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/ Institute for the award of any degree or diploma

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ACKNOWLEDGEMENT

I take this opportunity to express my sincere thanks and deep gratefulness to my supervisor Prof. (Mrs.) Susmita Mishra, Department of Chemical Engineering, NIT Rourkela for giving me this opportunity to work under her esteemed guidance. I feel indebted to her for her valuable advice and guidance throughout this research project.

I am thankful to Prof. P. Rath, HOD, Department of Chemical Engineering, NIT Rourkela for all the facilities provided during the course of my project.

I would also like to extend my sincere thanks to Mrs. Sandhya Rani Rebavarappu, Ph.D. Scholar for her help and guidance in the laboratory throughout the project. I would also like to thank my friend Sakariya Jigar Jayant, who was also working on a topic related to mine and proved to be a great help to me. It was overall a great experience working with all of them.

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ABSTRACT

The present study was conducted to isolate microorganisms capable of degrading phenol found in soil sample. The microorganisms were isolated from soil contaminated with phenol using enrichment technique and the isolates obtained from different soil samples were grown in nutrient broth, nutrient agar plates and mineral salt media. Mineral salt media was used for the degradation of phenol. The efficiency and resistivity of this biomass was checked for different concentrations of phenol with maximum of 1500 ppm. Three different microbes were identified and isolated which could resist this high concentration of phenol. The different characterization tests were performed on these three microorganisms. The ability of these microorganisms to degrade phenol at different pH was also observed. These microbes were also examined for their degrade ability by revealing them to different temperatures. The characterization tests and degradation study could give an identified microorganism able to degrade phenol. Outcome of this study offer a useful guideline in evaluating potential phenol degraders from the environment.

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Chapter 1

Introduction

1. INTRODUCTION

Due to presently increasing concern of pollution and its effect the pollution control methods are getting more attention and are equally more important because all the pollutants released in atmosphere are one or by other way very harmful to human in form of some or other disease or disorder. From the list of many pollutants one of the pollutant is phenol which is 11^{th} most toxic compound out of 126 toxic compounds given by EPA. Also the limit for inhalation of phenol vapor is set to 0.04ppm by EPA. This phenol released from industries in free form or in the form of phenol derivative is main problematic cause for pollution of soil and water from phenol release. Phenol which is released in the soil pollutes soil but microorganism in this soil can degrade phenol using phenol as their food. Hence the concept of degradation of phenol by using microorganism comes into picture. Degradation of phenol by utilizing microbial method is the most important methods because it does not produce any by products or any toxic waste. Microorganism is found to degrade phenol by two pathways mainly by ortho-pathway or meta-pathway and produces intermediates of tri carboxylic acids.

The growth of industries is in such a pace that that the pollution level is rising up with higher rates than its control measures. The industrial water is not only contaminating the surface water bodies but continuous dumping of this wastes in an open land leads these toxic pollutants from waste to seeps into the ground. As these pollutants seeps through the ground they also affect the ground water. People in the plateau region who depend on tube wells for drinking ground water get mostly affected by the contaminated ground water. This contamination contains the solid and liquid contaminants that are present in water. The contaminants may be biological, chemical or may be radioactive. These contaminants have devastating effects on our health and life of all the other species which are in their vicinity. They also have an adverse effect on the ecological system and lead to poison the food chains of terrestrial and aquatic life.

One more reason of pollution being taking place is the oil spills. It happens due to human negligence and it major form of pollution. Oil spills occurs when the petroleum products or crude oils are been exposed to environment by the pipeline breakage or vehicles or by industries. The animal that lives on the shores and nearby human being is mostly affected by these types of spills. This affects the marine life more strongly than any other ecosystems. The pollutants in

this oil or hydrocarbon are so toxic that can cause massive loss of species that live in the sea. Birds which are in the sea if they come in contact with this type of spills their feathers soaks the oil from the spills which leaves them paralysed. Also if the spills are in smaller quantity the oils finds its way into the fur and plumage of the birds. This makes it harder for them to float in water and hence they drown to death. These types of spill are also dangerous for the mammals because it restricts the ability of mammals to regulate their body temperature and die. This type of oil spills also leads to serious economic loss. Hence this type of pollution needs to be controlled with very efficient method.

Phenol is degraded by so many microbes which utilizes phenol as the sole carbon source for their growth. Several microbes both anaerobic and aerobic microorganisms degrading phenol are isolated and characterized, while microorganisms capable of aerobic phenol degradation were defined as early as 1908. *Pseudomonas putida* is the most widespread organism for the degradation of phenol amongst the various microorganisms. Along with bacteria, fungi are known for their multiplicity and notable ability in degrading phenolic compounds. Contrary to bacteria, fungi can grow under ecologically stressed environments such as low nutrient availability, low water activity and at low pH values where bacterial growing might be inadequate. *Trichosporon*, *Rhodotorula* species were able to utilize phenol as the only source of carbon and energy. *Fusarium flocciferum*, white fungi, *Phanerochaete* chrysosporium have also been shown to utilize phenols. Parameters for example contaminant concentrations, feasible biomass, concentrations, temperature, pH, microbial completion and adaptation are the most important parameters that affect phenol degradation rate and depends on the period during which the culture was adapted to phenol.

This thesis outlines the identification and characterization of phenol degrading organism from the contaminated soil.

Chapter 2

Literature Review

2. LITERATURE RIVIEW

2.1 PHENOL: TOXICITY AND PERMISSIBLE LIMIT

Phenol

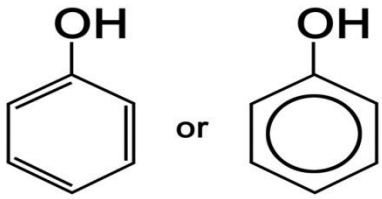


Fig2.1: Structure of Phenol

Phenol is an aromatic compound having one hydroxyl group attached to the benzene ring. It is a hygroscopic, crystalline solid with distinctive odor and is acidic. Phenol is very soluble in water and is quite flammable. Phenol is moderately volatile at room temperature. It has a very strong odor with an odor threshold of 0.04 ppm and a sharp burning sensitivity.

Table 2.1: Chemical and Physical properties of phenol

PROPERTY	PHENOL
Formula	C_6H_5OH
Molecular Weight(g/mole)	94.11
Water solubility(g/L at 25 °C)	87
Melting Point (°C)	43
Boiling Point (°C)	181.8

Toxicity

Acute exposure of phenol causes damage and disorder of central nervous system. It leads to collapse and progressively coma. A drop in body temperature occurs and this is known as hypothermia. Mucus membrane also shows very sensitive action to phenol. Muscular fits also occur. One of the effects is muscle weakness and tremors too. Extreme contact with phenol can

cause myocardial depression. A sensation of burning on skin also occurs due to phenol. Whitish effect and decay of skin may also result due to exposure of phenol. Phenol has an analgesic effect and causes gangrene. Exposure to phenol may result in irritation of eye, conjunctional swelling, corneal whitening and finally blindness. The continuous revelation phenol can induce renal damage. Other effects include fizzing from mouth and nose and subsequently headache. Chronic exposure may result in rashes, vomiting, and weakness; lose in weight, pain in muscles and nervous disorder. Due to muscle weakness, paralysis can be also being followed. It is also suspected that cancer and striation can also be caused. Phenol and its derivatives are toxic and classified as hazardous materials (Zumriye and Gultac, 1999)[6]. These phenolic compounds have various grade of toxicity and therefore their outcome in the environment is important. In recent years, a huge research work has been directed towards the development processes in which microbes are used to remove phenolic contaminants. Phenol is an antiseptic agent and is also used in surgery, which indicates that they are also toxic to many microorganisms (EPA, 1979).

2.2 MICROORGANISMS AND POLLUTION CONTROL

Whenever there is a spill of crude oil, the bacteria capable of degrading hydrocarbons proliferate quickly. The local community of the microbes is already adapted to the background supply of oil. It takes a certain amount of time—a lag time—for their population to increase in response to the influx of new resources. The flow of oil from a spill can temporarily beat the capacity of the local oil-degrading microorganisms. Eventually, along with the chemical and physical processes discussed above, the microbes will take care of the problem by consuming the oil compounds that are biodegradable. Microbes can be counted on to biodegrade oil over all time. However, the process might not be fast enough to prevent the ecological damage.

The process of biodegradation, defined as the use of microorganisms to eliminate pollutants is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry. Moreover, biodegradation is believed to be noninvasive and comparatively cost effective. Biodegradation by natural populations of microorganisms represents one of the major mechanisms by which petroleum and other hydrocarbon pollutants

can be removed from the environment and is relatively cheaper than the other degradation technologies.

2.3 EFFECT OF pH

Almost all the living thing right from microorganism to huge giants requires approximate neutral pH of 7 for their growth. Although the lowest pH or the most acidic limit allowable is found out to be 4 and the most basic pH of 9 hence beyond this limits no microorganism can survive as the acid and bases penetrates through the walls of cells interfering and disturbing the cell metabolism.

2.4 EFFECT OF TEMPERATURE

Availability of nutrients, pH, and temperature play major roles in the degradation of phenol. Here, we will see the effect of temperature in the degradation of organic pollutants. According to a research by Pakula et.al [63], there was seen a significant inhibition of phenol degradation at 30ºC. But, various studies on phenol degradation are being carried out in laboratory at an optimum temperature of 30 ºC. Some other studies described that described that when the temperature increased from 30°C to 34°C, no phenol degradation was perceived due to cell decay. All these studies show that the degradation of phenol is a process dependent on temperature. Growth rates roughly double for each 10°C rise in temperature within the usual mesophilic operational range from 10 to 30°C. Thus, one must make the conclusion to operate at the lower mesophilic range with an optimum temperature of around 35°C or in the thermophilic range with a temperature optimum of 55 to 60°C.

2.5 ENVIRONMENTAL POLLUTION CAUSED BY PHENOLIC WASTE

Phenol and its substituent compounds are the characteristics pollutants in the waste water generated from crude oil refineries, steel plants, coal conversion processes, manufacturing units of phenolic resins, pesticides and explosives, etc. Table 1.2 enlists the various industrial processes and the concentration of the phenol in the effluent generated from them.

Table 2.2 Phenol concentrations in industries effluents (BUSCA ET Al, 2008) [12]

Industry	**Phenol Concentration(mg/l)**
Coking operations	28-3900

Petrochemicals	2.8-1220
Coal Processing	9-6800
Refineries	6-500
Gas Production	4000
Pulp and Paper	0.1-1600
Pharmaceuticals	1000

2.6 METHODS OF TREATMENT FOR THE REMOVAL OF PHENOLIC WASTES

In view of phenol toxicity even at low concentration, it is very necessary to remove them before discharge of wastewater into water bodies. The applied treatment, which could be a single treatment or a combination of many treatments, must guarantee the removal of phenol to permissible discharge limits. The choice of treatment should depend upon the concentration, and volume of the effluent treated and cost of the treatment.

2.6.1 Physio-Chemical Methods for removal of Phenol

I) ION EXCHANGE: The removal of an ion from an aqueous solution by exchanging another ionic species is the basic principle of Ion exchange method.

II) EVAPORATION: The volatile hydrocarbons evaporate rapidly into the atmosphere when they reach the water surface.

III) DISPERSION: Dispersion is the process by which oil is broken down into small droplets and spread through the water, which facilitates microbial degradation.

IV) PHOTO-OXIDATION: Sunlight reacts with some oil constituents, especially the polycyclic aromatic hydrocarbons (PAHs). The process, known as photolysis, is important because by breaking aromatic ring structures, it develops the availability of such compounds to microbes and hence microbial degradation.

V) CHEMICAL OXIDATION: Chemical treatment involves the use of chemical agents to completely destroy or convert the contaminants to less toxic compounds, or intermediates that can be further degraded by microorganisms (Hamby D. M., 1996). Chemical oxidation of organic pollutants especially

phenol is a promising alternative when wastewater contains no biodegradable and/or toxic contaminants and also when the pollutant concentration is high.

LIMITATIONS OF THE ABOVE PROCESSES

The physio-chemical treatment technologies discussed above found to have drawbacks owing to the tendency to form secondary toxic intermediates and also proven to be costly (Klein and Lee, 1978; Talley and Sleeper, 1997). These processes are high energy consuming, non-economic and release effluents and waste waters which require further treatment and thus are disturbing for the environment.

2.6.2 BIODEGRADATION

Biodegradation is the breakdown of complex toxic organic contaminants to non-toxic and simpler elements by microbial activity. These contaminants can be considered as the microbial food source or substrate. Biodegradation of any organic compound is a series of biological degradation steps or a pathway that ultimately results in the oxidation of the parental compound that often results in the generation of energy. Microorganisms have the capability of degrading all naturally occurring compounds.

ADVANTAGES OF BIODEGRADATION

Biodegradation is being preferred over the other physio-chemical treatment methods of phenol due to its potential to degrade phenol completely and overcome the disadvantages postured by other processes .It is cost effective, produces no harmful products and most importantly maintains phenol concentration below the toxic limit. The microbes break down phenol completely and utilize it in the TCA cycle for energy production.

2.7 GROWTH KINETICS OF MICROORGANISMS

The amount of substrate must be sufficiently high as compared to the number of cells present to permit sufficient growth of the microbes. If the substrate concentration is low corresponding to the cell density, little or no increase in cells is possible. Hence it is known that the extent of growth depends on initial substrate concentration. In order to describe the kinetics of degradation

of substrate by microbes, several kinetic models such as growth-associated models (logarithmic, logistic and Monod with growth), non-growth associated models (zero order, first order and Monod based) and three-half order models have been reported in the literature (Schmidt et al., 1985; Brunner and Focht, 1986). To establish the effect of substrate concentration on the growth of microbial culture, specific growth rates of the culture at different substrate concentrations is calculated as per the following relationship:

$$\boldsymbol{\mu = \frac{1}{X}\frac{dX}{dt}}$$

Where μ is the specific growth rate (h^{-1}), X is the biomass concentration (mg/L). Usually, the microbial growth can be shown by a simple Monod equation:

$$\boldsymbol{\mu = \frac{\mu_{mS}}{S + K_s}}$$

Where, S is the limiting substrate concentration (mg/L), μ_{max} is the maximum specific growth rate (h-1), K_s is the half saturation constant (mg/L).

Rahul V. Hinge et al. (2014) have studied that phenolic compounds are found in wastewaters of various industries such as petroleum refining, coal conversion, plastics, textiles, iron and steel manufacturing as well as pulp and paper manufacturing. It is very important to remove phenols and aromatic compounds from polluted water before discharge into any natural water because of their toxicity to aquatic organisms. Conventional processes for removal of phenols from industrial wastewaters include extraction, adsorption on activated carbon, bacterial and chemical oxidation, electrochemical techniques.

Indu Nair et al. (2008) have studied degradation of phenol occurs as a result of the activity of a large number of microorganisms including bacteria, fungi and actinomycetes. Bacterial species include Bacillus sp, Pseudomonas sp, Acinetobacter sp, Achromobacter sp etc. Fusarium sp, Phanerocheate chrysosporium, Corious versicolor, Ralstonia sp, Streptomyces sp etc are also proved to be efficient fungal groups in phenol biodegradation. However, these microorganisms suffer from substrate inhibition at higher concentration of phenol, by which the growth is inhibited.

Bhavna V. Mohite et at. (2010) have studied that isolation of aerobic bacteria from soil contaminated with industrial xenobiotic compounds using enrichment technique containing phenol as the sole source of carbon and energy. The bacteria was isolated in pure culture and selected for their ability to degrade phenol. The soil bacterium was identified as *Streptococcus epidermis* coded as (OCS-B). The selected microbial strain can be effectively used for bioremediation of phenol contaminated sites as was able to degrade phenol up to 200mg/l which was also confirmed by HPLC analysis. Degradation intermediate compounds were also determined.

Paula M. et al. (1998) studied three novel nitrate-reducing microorganisms that are capable of using phenol as the only source of carbon was isolated and characterized. The three strains were presented to be different from each other based on physiologic and metabolic properties. The identification of the organisms was needed. Although analysis of membrane fatty acids did not result in identification, but the results of the fatty acid profiles were found to match with those of *Azoarcus* species. The results of this study add three new members to the genus *Azoarcus*, which previously comprised only nitrogen-fixing species associated with plant roots and denitrifying toluene degraders.

Abdul Haleem et al. (2013) studied to take advantage of the potential of locally isolated indigenous soil bacteria to degrade phenol. The isolation was conducted for all the soil samples and the isolates obtained from different soils were grown on nutrient agar plates, nutrient broth and mineral salt media. Degradation of phenol was done with mineral salt media. Growth of biomass along with phenol degradation was studied along with dry cell weight. The locally enriched and isolated soil bacteria were made potent up to 2.5 g/100 mL phenol concentration. These bacteria were examined for their degrade ability by maintaing different temperatures.

Chapter 3

Materials and Methods

3 MATERIALS AND METHODS

3.1 COLLECTION OF SAMPLES

Soil samples were collected from garage, Rourkela (say sample S1), and form back post cycle stand, NIT Rourkela (say sample S2). Isolation of microorganisms was then carried out in the biochemical laboratory.

3.2 MEDIA COMPOSITION AND CULTURAL CONDITION

Following media were used for the growth of microorganisms.

3.2.1 Nutrient Broth

Broth media consists of following compositions in 100mL distilled water.
Beef extracts 0.3 g, Peptone 0.5g, and NaCl 1g.

3.2.2 Nutrient agar

Nutrient agar consists of following composition.
Beef extracts 0.3g/100mL, Peptone 0.5g/100mL, Agar 1.5g/100mL and Distilled water 10mL.

3.2.3 Mineral salt medium

Following components are the basic ingredients in mineral salt medium (Banerjee et al., 2001): K_2HPO_4 (1.5g/L), KH_2PO_4 (0.5g/L), NaCl (0.5g/L), NH_4NO_3 (1g/L), Mg $SO_4.7H_2O$ (0.5g/L), $FeSO_4.7H_2O$ (0.01g/L), $CaCl_2.2H_2O$ (0.01g/L) and NH_4SO_4 (0.5g/L).

3.3 ISOLATION OF BACTERIA DEGRADING PHENOL

We have to isolate microorganisms for the degradation of phenol for which we need to first grow the microbes with the help of nutrient broth which is food for the microbes. The process of providing the microbes with a specific media for their growth is called enrichment. This process will then be followed by survival of microorganisms in different concentration of phenol to check the maximum concentration up to which microbes can survive.

3.3.1 Enrichment process

Enriched media consist of nutrients required to support the growth of a variety of organisms and they are commonly used to grow as many different type of microbes as present in the specimen, in this case soil samples. This is basically the growth of microorganisms.

The following is the procedure done for enrichment process:

- Sterilization of water contained in a conical flask is done in autoclave to avoid any contaminants which includes heating at 121°C for 15 minutes.
- 1 g of the soil sample was mixed with 9 ml of the sterilized water and was mixed thoroughly.
- Nutrient Broth media was prepared by dissolving 1.3 g of nutrient broth in 100 ml of water and was sterilized in autoclave.
- 1 ml of the soil sample was added to the nutrient broth media after cooling in laminar flow hood which offers cooling free of any contamination in the presence of UV light.
- This media was then sealed with cotton plug and paraffin tape to avoid any contamination.
- It was then kept in an incubator at a temperature of 30°C and shaker at an rpm for 24 hours.
- This was done for both the samples of soil.

It was observed that there was sufficient growth of biomass in the nutrient broth which could be inferred by the turbidity of the sample.

3.3.2 Microorganisms resisting phenol

We now have an enriched culture which contains all the microorganisms which was present in the soil. The growth of microorganisms which can survive in the presence of phenol is to be checked. The growth in phenol can be checked if phenol is the only source of carbon for the microbes for which we need to cut the supply of all other sources of nutrients. The minimal salt media along with the different concentration of phenol is now the source for the growth of microbes.

The following are the steps done for the same:

- 100 ml distilled water was taken in 250 ml flask and the different salts as per the minimal salt media composition were added to make 100 ml media.
- The stock solution of 5000 ppm phenol solution was prepared and stored.

- 100 ppm solution was prepared by adding 10 ml of stock solution to 90 ml of the media solution.
- This solution was then sterilized and was kept in laminar flow hood for cooling.
- 1 ml of the culture was added to the 100 ppm solution and this is called inoculation.
- This solution was then sealed and was then incubated for 24 hours at the same condition mentioned above.

The growth of biomass was seen the next day (turbidity) which conclude that the microorganisms could resist 100 ppm of phenol as they used phenol as their carbon source.

The same procedure was repeated for concentration from 100 ppm to 1000 ppm with an increment of 100 ppm each day and at different incubation temperatures (25°C, 30°C, and 40°C).

3.4 ISOLATION OF SPECIFIC MICROORGANISM

The media of different ppm concentrations contain variety of microbes. We need to isolate a specific microorganism which can resist phenol and we need to identify that specific microorganism. There are various methods for the isolation of specific microorganisms.

The following are the various methods for isolation techniques:

3.4.1 Serial Dilution Method

A serial dilution is a series of sequential dilutions used to reduce a dense culture of cells to a more usable concentration. Each dilution will reduce the concentration of microorganisms by a specific amount.

The following is the procedure followed:

- 1 ml of the original culture was taken with the help of a pipette.
- It was then poured in a test tube containing 9 ml water.
- It was then again transferred from test tube 1 to test tube 2 and then from test tube 2 to test tube 3.
- These cultures were then used for inoculating in pour plate method which we will discuss further.

This same procedure was done for 1000 ppm as well as 1500 ppm to reduce the concentration of biomass so that it can give less dense colonies.

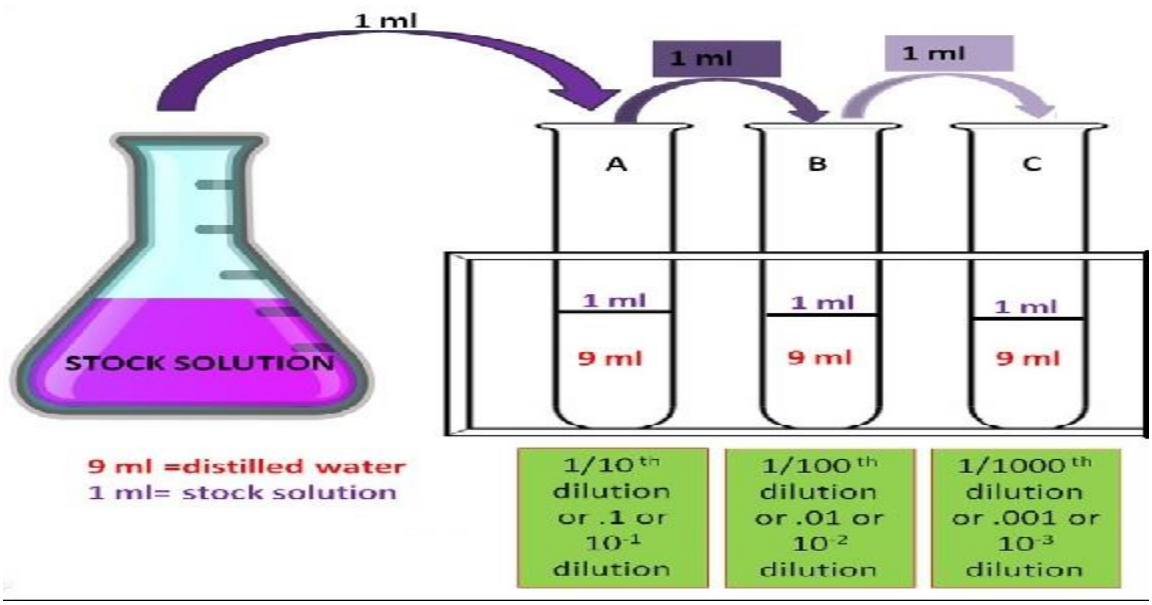


Fig.3.1 Serial Dilution Method

3.4.2 Pour Plate Method

This method basically involves spreading of culture on a solidified media so that growth of colonies can take place. It would then be easy to isolate and then store that particular colony for future use and for the study of degradation of phenol.

The following procedure was followed:

- 2 grams of agar powder with 1.3 grams of nutrient broth was mixed in 100 ml of distilled water.
- The petri plates and the agar media were then sterilized in autoclave at 121°C for 15 minutes.

For 100 ppm, 200 ppm, 300 ppm, 500 ppm:

- 0.5 ml of the culture was spread on the plate and then the agar media was spread on top of that.

For 1000 ppm and 1500 ppm:

- 0.5 ml of the original culture as well as the 3 cultures obtained from serial dilution technique was spread on different petri plates and then the agar media was spread on them.
- The agar media was then allowed to cool and solidify.

- The plates were then sealed with paraffin tape and were kept for incubation at 30°C for 24 hours.

3.5 MAINTENANCE OF PURE CULTURE

The specific colonies obtained needs to be transferred only to nutrient broth so that this pure culture can be stored. This pure culture containing only a single type of microorganism will then be used for phenol degradation. The method followed for long term storage is slanting followed by streaking as explained below.

3.5.1 Streaking Method

The streak plate method is a rapid qualitative isolation method. The techniques commonly used for isolation of discrete colonies initially require that the number of organisms in the inoculums should be reduced. It is essentially a dilution technique that involves spreading a loopful of culture over the surface of an agar plate. The resulting decrease of the population size ensures that, following inoculation, individual cells will be sufficiently far apart on the surface of the agar medium to effect a separation of the different species present. Although many type of procedures are performed, I performed quadrant streak and pour plate streak.

3.5.2 Slant Preparation Method

A culture made on the slanting surface of a solidified medium in a test tube that has been tilted to provide a greater area for growth and also for long term storage.

The following procedure was followed for the preparation of slants:

- 2 grams agar powder and 1.3 grams of nutrient broth were mixed in 100 ml distilled water taken in a conical flask.
- This flask and empty test tubes were then covered and sterilized in autoclave at 121°C for 15 minutes.
- The media was poured into test tubes and kept in a slant position and allowed to cool and harden.
- The selected colonies were then transferred into the respective slants using the streaking loop.
- The streaking loop was then applied on the media in a zig-zag pattern.
- The test tubes were sealed with cotton plug and paraffin tape and were stored in refrigerator.

The same method can also be done in petri plates but slants were preferred as this method avoids contamination. The slants also take less space for storage and it is easy to transfer from slants.

3.6 GROWTH KINETICS OF MICROORGANISMS DEGRADING PHENOL

The procedure followed to determine the growth kinetics of microorganisms is as follows:

- The microorganisms isolated were also inoculated in broth media and stored.
- 50 ml minimal salt media of 1500 ppm phenol concentration was prepared.
- It was then sterilized in autoclave and allowed to cool.
- 1 ml of the culture from the broth media was taken and was added to the solution.
- It was then kept for incubation at 30°C and 100 rpm.
- The culture was taken out after 6 hours and 1 ml of the culture was taken in an Eppendorf tube.
- The tube was then kept in the centrifuge at 1400 rpm for 10 minutes.
- It was observed that biomass was collected at the bottom and the top liquid part is called the supernatant.
- The supernatant was then separated from the top and the biomass was allowed to dry for 15 minutes.
- .The weight of an empty Eppendorf tube was measured and then the weight of the tube with biomass was measured.
- The difference of these two weights gave the growth of biomass resulted due to phenol degradation.

3.7 CHARACTERIZATION METHODS

The isolates were identified based on biochemical characterization. Many tests have been known over years for the classification of microorganisms into families, genera, species and even sub specie. Some of these tests are simple which can be performed in our laboratory and some require equipment and chemicals which are not present in our laboratory. The tests done in the laboratory involves Gram Staining test, Amylase test, Starch hydrolysis test, Urease test, Catalase test and Mannitol test. These tests give different characteristics of the isolates. The tests are mentioned below with their respective procedures and significance.

3.7.1 Gram Staining

This test is done to differentiate between gram positive and gram negative organisms. Gram positive organisms contain a thick layer of protein-sugar complex called peptidoglycans in their cell wall whereas for gram negative, it only comprises of 10-20% of the cell wall.

The procedure is as follows:

- It was started with a fixed bacterial smear on slide.
- The slide was flooded with crystal violet stain as primary stain.
- It was rinsed with water after 1 minute and then the slide was flooded with iodine.
- It was again rinsed with water after 1 minute.
- It was then flooded with acetone alcohol as decolorizer and rinsed with water after 15 seconds.
- It was rinsed with safranin as secondary stain and again rinsed with water after 1 minute.
- Then the slide was blot dry and observed under microscope.

Gram positive bacteria will stain purple.

Gram negative bacteria will stain red/pink.

3.7.2 Amylase Test

This test is done to determine if the organism is capable of breaking down starch into maltose through the activity of the extra-cellular α-amylase enzyme.

The procedure followed was;

- A sterile loop was used to pick up a few colonies of the pure culture from the slant. Then it was streaked across a starch plate in the form of a line across the width of the plate.
- It was then incubated at 37°C for 48 hours.
- 3 drops of iodine were added on the colonies and result was recorded after 15 minutes.

Clear around colonies: positive

Purple/blue around colonies: negative

3.7.3 Catalase Test

This is used to test for the presence of the enzyme catalase.

A loopful of colony of pure culture was picked up and kept on a clean glass slide. 2 drops of 3% H_2O_2 was added and waited for 15 seconds.

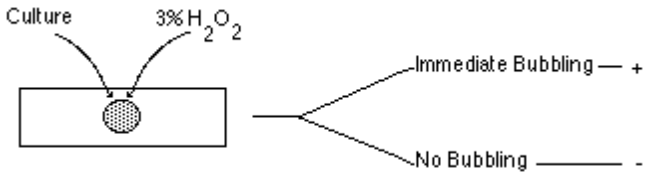


Fig.3.2 Catalase Test analysis

Positive test: immediate bubbling (O_2 formed)

Negative test: no bubbling

3.7.4 Mannitol Test

To determine the ability of an organism to grow in 7.5% NaCl and ferment mannitol.

A Mannitol salt agar plate was streaked with a light line of inoculum from the pure culture of the test organism using a sterile loop. It was then incubated at 30°C for at least 48 hours.

Any significant growth says that the organism is a Staphylococcus species.
Positive: Growth, yellow color (mannitol "+").
Negative: Growth or no growth; red or orange color (mannitol "-").

3.7.5 Urease Test

The urease test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide.

The surface of a urea agar slant was streaked with a portion of well isolated colony. The cap was left on loosely and incubated the test tube at 35°C for 48 hours.

Positive: Color changes from light orange to pink. (Organism produces urease)

Negative: No color change.

Chapter

Result and Discussion

4. OBSERVATIONS

Here, we will see the observations and record the results of each of the above procedures discussed above. It starts with the growth of the microorganisms from the soil media, then its resistance in different concentrations of phenol and also at different incubation temperature for a particular concentration, isolation of specific microorganism, growth kinetics and the results of different characterization tests.

4.1 ENRICHMENT IN DIFFERENT MEDIA

The enrichment process was performed for two soil samples S1 and S2 and in two different Medias, i.e. LB media and nutrient broth media to check the growth of microorganisms.

Table 4.1 Enrichment in different media

Soil Samples	**Nutrient Broth**	**LB media**
Sample,S1	Growth	No growth
Sample, S2	Very less	No growth

It was observed that there was a growth of microorganisms for both the soil samples in nutrient broth media and that too there was more growth (more turbidity) for sample 1, hence the culture obtained from sample 1 was preferred and further processes were performed with nutrient broth as their source of energy as it was observed that microorganisms could adapt that media better than the LB media.

4.2 GROWTH OF MICROORGANISMS RESISTING PHENOL

The experiment was performed to observe the growth of microorganisms with different concentrations of phenol in minimal salt media and phenol as the only carbon source. The incubation temperature was also varied to check the optimum temperature for their growth. The growth with the variation of temperature and concentration is given in table 4.2. The sign “+ve” indicates that the microbes could resist the concentration in that particular temperature and the sign “-ve” implies just the opposite.

Table 4.2 Growth of microbes resisting phenol in MSM

Concentration of phenol (in ppm)	**Growth at different temperatures**		
	25 °C	**30 °C**	**40 °C**
100	+ve	+ve	-ve
200	+ve	+ve	-ve
500	+ve	+ve	-ve
700	-ve	+ve	-ve
1000	-ve	+ve	-ve

Fig 4.1 Growth of microorganisms at different concentration at 30°C

The results obtained indicate that there was some growth at lower concentration for 25°C but still this temp is quite low for their survival they could not at all survive at 40°C. This indicates that 40°c is quite high for their survival at every concentration. Therefore, it was observed that 30°C is the optimum temperature for the microorganisms that we have enriched.

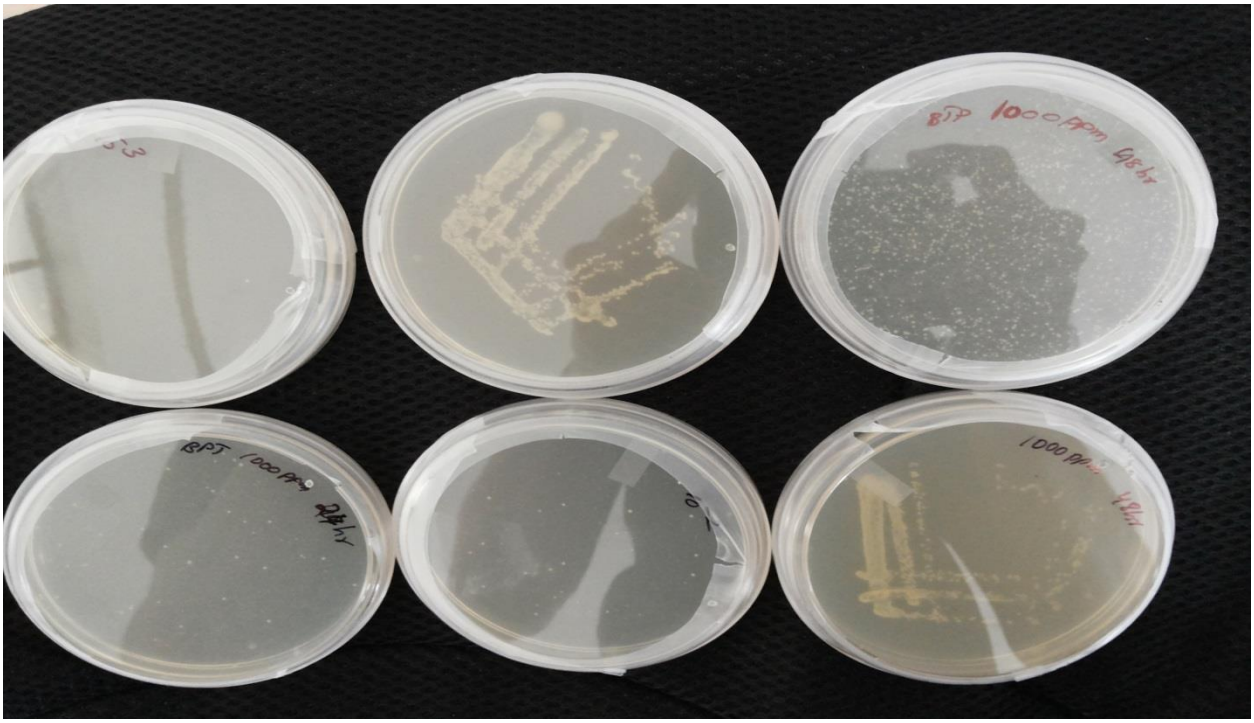
4.3 ISOLATION OF SPECIFIC COLONIES OF MICROORGANISM

The isolation of specific colonies of microorganisms was done by serial dilution method followed by pour plate method as explained above. The cultures at different concentrations were first subjected to 1/1000 times concentration and then the original culture along with the diluted culture was spread in pour plates.

Table 4.3 Isolated Colonies at different concentration

Concentration of phenol (in ppm)	**Structure of colonies by Serial Dilution Method**			**Type of Colonies**
	10^{-1}	**10^{-2}**	**10^{-3}**	
100	Mat	Mat	Mat	Mat
500	Mat	Mat	Mat	Mat
700	Mat	Mat	Mat	Mat
1000	More than 10	>10	7	5
1500	More than 10	3	No colonies	3

Fig 4.2 Isolated Colonies at 1000 ppm



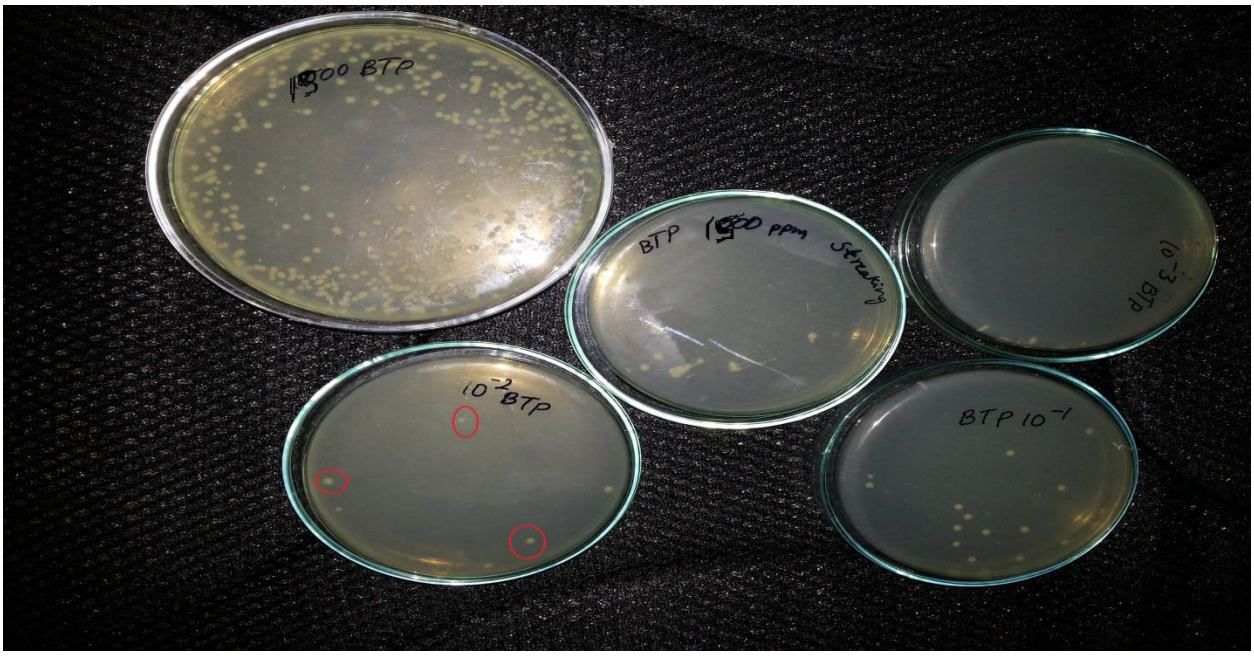


Fig 4.3 Isolated colonies at 1500 ppm

We performed the serial dilution up to 1000 ppm but there were no specific isolated colonies but rather crowded colonies as seen in Fig 4.2, so to reduce the number of colonies, the serial dilution was also performed for 1500 ppm and then three isolated colonies were observed for 1/100 dilution. These three colonies, each colony containing a single type of organisms or say PR1, PR2, PR3 were isolated as seen in Fig.4.3 and stored in slants.

Colony 1 containing a single type of microorganisms resisting phenol: **PR1**

Colony 2 containing a single type of microorganisms resisting phenol: **PR2**

Colony 3 containing a single type of microorganisms resisting phenol: **PR3**

These three colonies were then stored in each slants for long term storage and to study growth kinetics of microorganisms and degradation of phenol. The streaking method in slant was followed as explained above. The microorganisms stored in slants can be seen in Fig.4.4.

Fig.4.4 Storage of the isolated microorganisms in slants

4.4 GROWTH KINETICS

The cell growth at different time with an interval of 6 hours was recorded. The readings are as per noted in Table 4.4. A graph was plotted with time versus the biomass growth. The maximum growth of biomass shows that particular microorganism can resist phenol with maximum growth and maximum degradation of phenol. It is observed from the table below that both the organisms PR3 and PR2 show maximum growth at 24 hours but the growth of biomass is greater for the organism 3. The plot the organism 3 shows a peak at 24 hours. PR1 shows maximum growth at 36 hours.

Table 4.4 Observation for the cell growth with respect to incubation time for three isolated colonies

Time(in hours)	Cell growth or biomass(in mg)		
	PR1	**PR2**	**PR3**
0	0	0	0
6	0.1	0.045	0.045
12	0.19	0.15	0.18
18	0.22	0.185	0.185
24	0.34	0.35	0.6
30	0.49	0.34	0.57
36	0.32	0.28	0.3
42	0.16	0.16	0.18
48	0.02	0.035	0.05

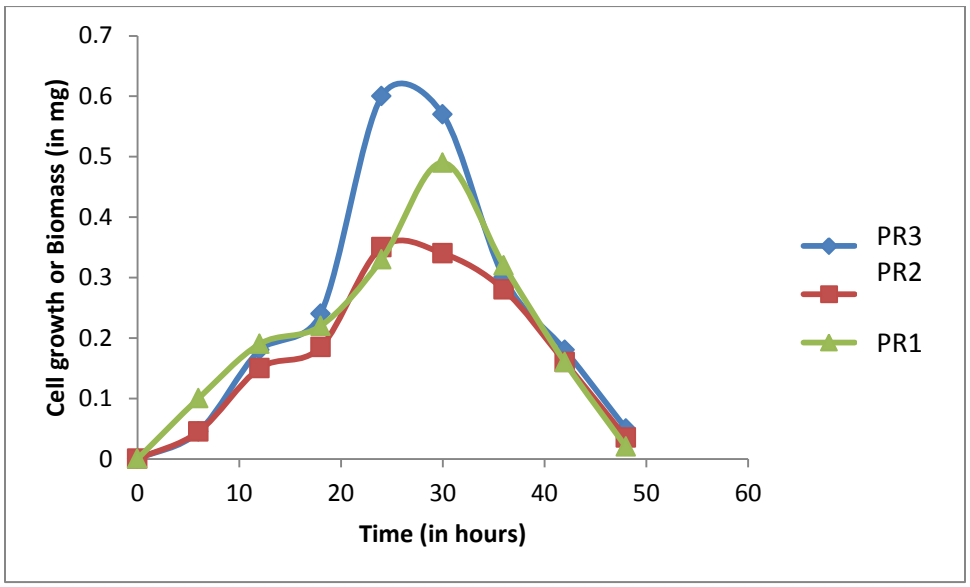


Fig.4.5 Variation of cell growth with time

Then the effects of pH and temperature variation on the cell growth were studied for all the three organisms and the readings recorded are shown in table 4.5 and table 4.6.

Table 4.5 Effect of pH on cell growth

Organism	**Biomass at different pH (in mg)**				
	5	**6**	**7**	**8**	**9**
PR1	0.28	0.31	0.39	0.45	0.41
PR2	0.25	0.29	0.34	0.32	0.31
PR3	0.33	0.38	0.55	0.42	0.34

The organism PR3 shows maximum growth in neutral media.

Table 4.6 Effect of temperature on cell growth at their optimum conditions

Organism at their optimum condition	**Biomass at different temperatures (in mg)**		
	25°C	**30°C**	**40°C**
PR1	0.36	0.42	0.38
PR2	0.29	0.33	0.31
PR3	0.48	0.56	0.42

30°C is the optimum temperature for all the three microorganisms.

4.5 CHARACTERIZATION RESULT

Table 4.7 Results of the different characterization tests for the three organisms

Characterization Tests	**Microorganisms**		
	PR1	**PR2**	**PR3**
Gram Staining test	+ve	+ve	+ve
Amylase test	+ve	+ve	+ve
Catalase test	-ve	+ve	+ve
Mannitol test	-ve	-ve	+ve
Urease test	-ve	-ve	-ve

These tests show the various characteristics of the three organisms. The objectives behind these tests are already stated in the tests definition. The positive test indicates that the organism responds to that test and vice versa.

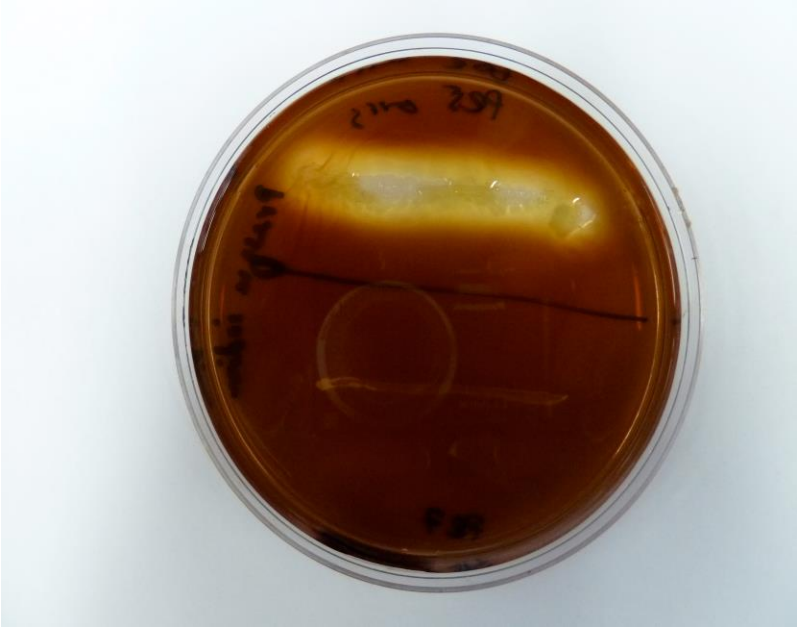


Fig 4.6 Positive amylase test for PR3

4.6 DISCUSSION

This project aimed at finding a particular microorganism responsible for degrading phenol. We could enrich soil and had the growth of microorganisms from soil sample and the other sample could not get adapted to both the medias. The isolation was to be checked for a wide range of concentration of phenol as the microorganism was able to resist concentration as high as1500 ppm. Sometimes growth could not be found at all due to the mistakes in media preparation, so care needs to be taken while preparing the media for the pour plates. Finally, 3 isolated colonies were obtained at concentration of 1500 ppm diluted to 100 times or 10^{-2} concentration.

Then, our work was to choose the best among the three organisms obtained means the one with greater resistant to phenol or greater degradation potential. The growth kinetics showed that the organism PR3 did the maximum degradation and was more resistant to phenol. Then the focus shifted only to this organism. Its growth was then checked at different temperatures which result in maximum growth at around 30°C which is a temperature easy to maintain, so economical. It could survive at neutral pH. The various characteristics tests were performed which gave different characteristics. So this PR3 responded positive to all tests which was much more advantageous. The positive response to mannitol test could give us an idea that this organism was of staphylococcus species. But, the exact name of the organism was needed to be known. Some expensive equipment are used for that which were not supported in our laboratory. Therefore, the organism was sent to NCIM, Pune for its tests and identification.

Chapter 5

Conclusion

5. CONCLUSION

This project starts with growth of microorganisms and ends with identification of that particular microorganism. We now conclude that the entire procedure could be completed successfully with a valid outcome. The soil could be enriched giving proper growth of the microorganisms as a result we were able to isolate the specific colony of microorganism degrading phenol. The organism PR3 was found to be the best among the three organisms isolated on the basis of growth kinetics and degradation capability. The characterization tests gave the mannitol positive test indicating it to be of staphylococcus species. The further research technique and various tests resulted in the identification of the organism PR3 as ***staphylococcus lentus.***

This is the organism responsible for degradation of phenol and could be further used in biodegradation techniques.

Chapter 6

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