Treatment of aromatic hydrocarbon containing solution using marine microorganisms

Thesis
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Declaration of Originality and Compliance of Academic Ethics

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of her ***Master of Bioprocess Engineering*** studies during academic session 2017-2019**.** All information in this document have been obtained and presented in accordance with academic rules and ethical conduct.

I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are fully authentic to this work.

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Thesis Title: ***Treatment of aromatic hydrocarbon containing solution using marine microorganisms***

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CERTIFICATE

*This is to certify that the thesis entitled "**Treatment of aromatic hydrocarbon containing solution using marine microorganisms**" has been carried out by Nikhil Kumar Singh in partial fulfilment of the requirements for the degree of Master of Bioprocess Engineering from Jadavpur University, Kolkata is recorded as bona fide work that has been conducted under the joint supervision of Prof. (Dr.) Debashis Roy and Prof. (Dr). Papita Das. The contents embodied in the thesis have not been submitted to any other university for the award of any degree or diploma.*

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Abstract

In this era of urbanization and utter industrialization there has been rapid increase in generation of waste that directly introduces into the water bodies, affects nature as well as mankind directly or indirectly. Industrial waste produced through the industrial process is highly toxic and needs to be remediate before being discharged into the natural water bodies such as lakes, streams, ponds, and oceans. Poly-aromatic hydrocarbon such as phenol and their derivatives are secrets from the industrial plants usually introduces directly into the water bodies. There are several physico-chemical methods like adsorption, chemical oxidation extraction etc. are introduces to remediate the waste water but bioremediation using microbial catalyst method is more suitable and cost effective. These marine microorganisms are capable of degrading the highly toxic phenolic compounds and convert into smaller non-toxic molecules like CO_2 and H_2O. In this study, we used fungi and bacteria to remediate the waste water. Pre-isolated fungi are screened by their degradation capabilities and characterized by nucleotide homology as *Alternaria alternata* and *Aspergillus niger*. The degradation assay suggested the fungal strain is capable of using the phenol as the carbon and energy source. It has been investigated that mycelium of *Alternaria alternata* are capable of degrading the phenol concentration up to 300 mg/L while *Aspergillus niger can* degrade phenol up to 500mg\L using pure culture. We have also investigated degradation capabilities of unidentified bacteria collected from Radhanager, Andaman and Digha, West Bengal. It can degrade phenol concentration up to 500ppm. Unidentified bacterial species are isolated and screened under variable phenol concentration to check their degradation capabilities. Removal of phenol from wastewater was studied at various conditions like pH, temperature, incubation period, and shaker's rotational speed to optimized process parameter for both species. Response Surface Methodology (RSM) was studied for optimization of process parameter for both fungi. Biochemical characterizations were studied using various biochemical tests such as Carbohydrate utilization test, Malonate utilization Test, Phenylalanine deamination test, Starch Hydrolysis test etc. for unidentified bacteria species isolated from Radhanager, Andaman and Digha, West Bengal.

Key words: Phenol, bioremediation, Alternaria *alternata*, Aspergillus *niger*, *Response Surface Methodology (RSM)*

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Contents

Chapter 1.

Introduction

Water is the most valuable natural resource that exists on our planet. It covers 70% of the earth's surface and without this invaluable compound; the life on the Earth would not exist. This is a widely recognized fact; pollution of water resources is a common problem that is being faced. The pollution in the environment with man-made (synthetic) organic compounds has become a major problem with urbanization and extensive industrialization, (Ghisalba, 1983). The industrial and domestic activities have polluted the surface water as well as ground water to a greater extent (Armour, 1991). The natural water bodies are contaminated by various toxic pollutants. In addition to having adverse health implications, wastewater contamination also has natural and ecological effects. Toxic wastes are being released into the environment, causing extensive environmental contamination such that many of our natural water reserves are damaged beyond repair (Ollis, 2000). Due to discharge of toxic effluents long-term consequence of exposure can cause cancer, delayed nervous damage, malformation in urban children, mutagenic changes, neurological disorders etc. (Govindarajalu, 2003).Nevertheless, to ensure sustainable quality of life the environmental impact of these activities must be minimized. While conservation and better utilization of resources have the greatest influence on sustainability of the planet; reduced generation, improved treatment technology and utilization of wastes are the best techniques for the maintenance of the environmental quality.

Petroleum and refinery waste water, hereafter termed as 'petroleum waste water', originate from the effluent of industries primarily involved in exploration of crude oil , refining crude, oil and manufacturing fuels lubricants and other petrochemicals intermediates (Harry,1995). During the refining process of crude oil, a large amount of waste is consumed and consequently, considerable volume of water are generated from the petroleum industries (Coelho etal. 2006). It has been estimated that the volume of petroleum refinery effluent generated during processing of crude oil is 0.4-1.6 times the amount of crude oil process (Coelho etal. 2006). Hence, total of 33.6 million barrels per day (bpd). Of effluent is produced worldwide, based on the present yield of 84 million bpd of the crude oil (Doggett and Roscoe, 2009).

The industrial process generate a variety of waste molecules such as toxicity, carcinogenic and mutagenic properties which pollutes air and water and hence introduces negative impact (Busca et al. 2008). The main constituents produces from the coal conversion processes, herbicide manufacturing, coke ovens phenolic resin manufacturing, petroleum refineries, petrochemicals and fiberglass manufacturing is phenol. (El-Ashtoukhy et al. 2013; Veeresh et al. 2004; Jadhav and Vanjara 2004).). Phenol and its derivatives are a major source of environmental pollutants (Said et al. 2013; Varma and Gaikwad 2008). Phenol is one of

the pollutant produces from industrial processes that introduces into the aquatic ecosystem gradually affects the indigenous biota such as algae, vertebrates and invertebrates. (Babich and Davis 1981). The concentrations of these compounds can range from one to several hundred mg/L (Moussavi et al. 2008). Industrial wastewaters associated with the manufacture of halogenated organics characteristically have concentrations as high as hundreds of mg/L (Annachatre and Gheewala 1996).organic and inorganic effluents discharge in the water body is one of the great public concern. (Pradeep et al. 2014). Their fate in the environment is of great importance as they are toxic, recalcitrant and bioaccumulation in organisms (Annachatre and Gheewala 1996). Phenol is one of the 129 specific priority chemicals that is considered toxic under the 1977 Amendments to the Clean Water Act and for which the US Environmental Protection Agency (EPA) has issued water quality criteria adversely affects the biota. (Singh et al. 2013; Babich and Davis 1981). Low concentration of phenol as 5 mg/L imparts typical smell upon chlorination and the World Health Organization (WHO) prescribed 1 mg/L as the maximum permissible concentration of phenol in drinking water (Saravanan et al. 2008).

The field of microbial ecology is advancing rapidly. Recent technological advances have dramatically expanded the scope and accuracy of scientific techniques for ability to degrade poly aromatic hydrocarbons (PAHs). Most research in microbial ecology has emphasized the micro, even the molecular, over the macro. A thorough understanding of the ecology directly impacting the formation of the microorganisms in environment would be is necessary for the proper management to human benefit. Thanks to decades of molecular research, it is now possible for microbial ecologists to understand the relationship between the microscopic individual microorganisms and the systemic biogeochemical cycles those organisms drive.

The physiological ability of certain microbes to metabolize and degrade PAHs is of great interest to the scientific community. Microbes able to completely metabolize and remove PAHs could be incorporated into inexpensive strategies for the passive remediation of contaminated sites. One of the most widely used in the organic compounds in existence in the environment is phenol. It is a basic structural unit for a variety of synthetic organic compounds including agricultural chemicals and pesticides. Extensive research work has been done in field of biodegradation and several microorganisms are found which have the capability to degrade phenol. Many Microorganisms such as fungi bacteria, and actionmytes degrade phenol to a large extent. Fungi species such as *Fusarium sp, Coriousversicolor, Phanerocheate chrysosporium, Streptomyces sp, Ralstonia sp* etc. *Pseudomonas sp, Bacillus sp, Achromobacter sp Acinetobacter sp etc*. are the researched bacteria in the phenol removal. Hence in our study we will use the biodegradation method to degrade phenol by microorganisms collected from the contaminated sight and

optimum conditions will be compared for these microorganisms and the best one will be found and characterized.

Drainage of the municipal or industrial sewage to surface water results as penetration of ecosystems from the phenol and its derivatives. . The pollution of the aquatic environment by phenols could modify the biota of this environment because most of these compounds exhibit a high degree of toxicity (Lika and Papadakis 2009).

There are several ways to remove phenol from the industrial effluent or from the phenol contaminated water this methods are other than the biodegradation method for phenol. Phenol has various effects on human being both short term and long term. Various methods use for phenol separation are electro coagulation, extraction, adsorption and ion exchange, advanced oxidation process, electro-fenton method, membrane separation, photodecomposition etc. This all methods are used for effective phenol separation. Adsorption is used extensively to remove phenol from effluent waste. It was determined that the particle size and flow rate are the cause for sorption of phenol, and if we increase flow rate and particle size the particle size and break time point also decreases. If adsorbent is used 89-97% removal of phenol can be guaranteed .Other method is electro-coagulation it is basically based on the electro chemical removal of compounds. This method has been adopted by many petroleum industries to remove phenol. But these all processes are very expensive and difficult to handle. Biological degradation of phenol by using microorganism is such a technique to remove phenol from phenol contaminated water. This process unlike the above ones is toxic free process it has no by products and more importantly it is cost effective process. This process can be handled easily with less equipment and is economically viable.

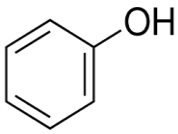
Chapter 2.

Literature Review

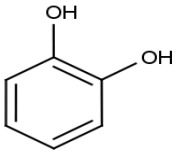
2.1 Xenobiotics:

Xenobiotic compounds are man-made chemicals that are present in the environment at high concentrations and are highly toxic in nature. These compounds normally have unusual chemical or physical properties that make them refractory to biodegradation. The principal xenobiotic include drugs, pesticides, carcinogens and various compounds that have been introduced into environment by artificial means, which resist to normal decomposition and persist for longer time in the environment.

Among all the toxic compounds, phenol and its substituent phenolic compounds contribute a remarkable adverse impact to the environment. These are major xenobiotics, which are often found in the effluents discharged from the industries such as paper and pulp, textiles, gas and coke, fertilizers, pesticides, steel and oil refineries etc., (Ghadi and Sangodkhar, 1995; Mahesh and Rama, 1999). During the last two decades, phenolic compounds have become the subject of intense research in the preservation of our environment. The US Environmental Protection Agency (EPA, 1979) had classified the Phenolic compounds as high priority pollutants due to their extensive impact on the deterioration of the water environment.



Phenol



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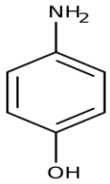
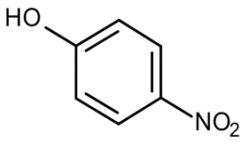


P-aminophenol



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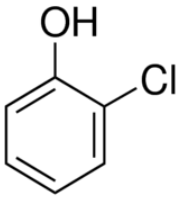
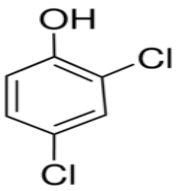
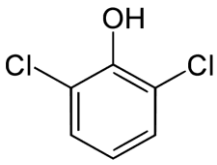


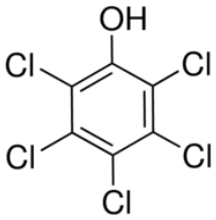
2-chlorophenol



2,4 dichlorophenol



2,6 dichlorophenol



pentachlorophenol

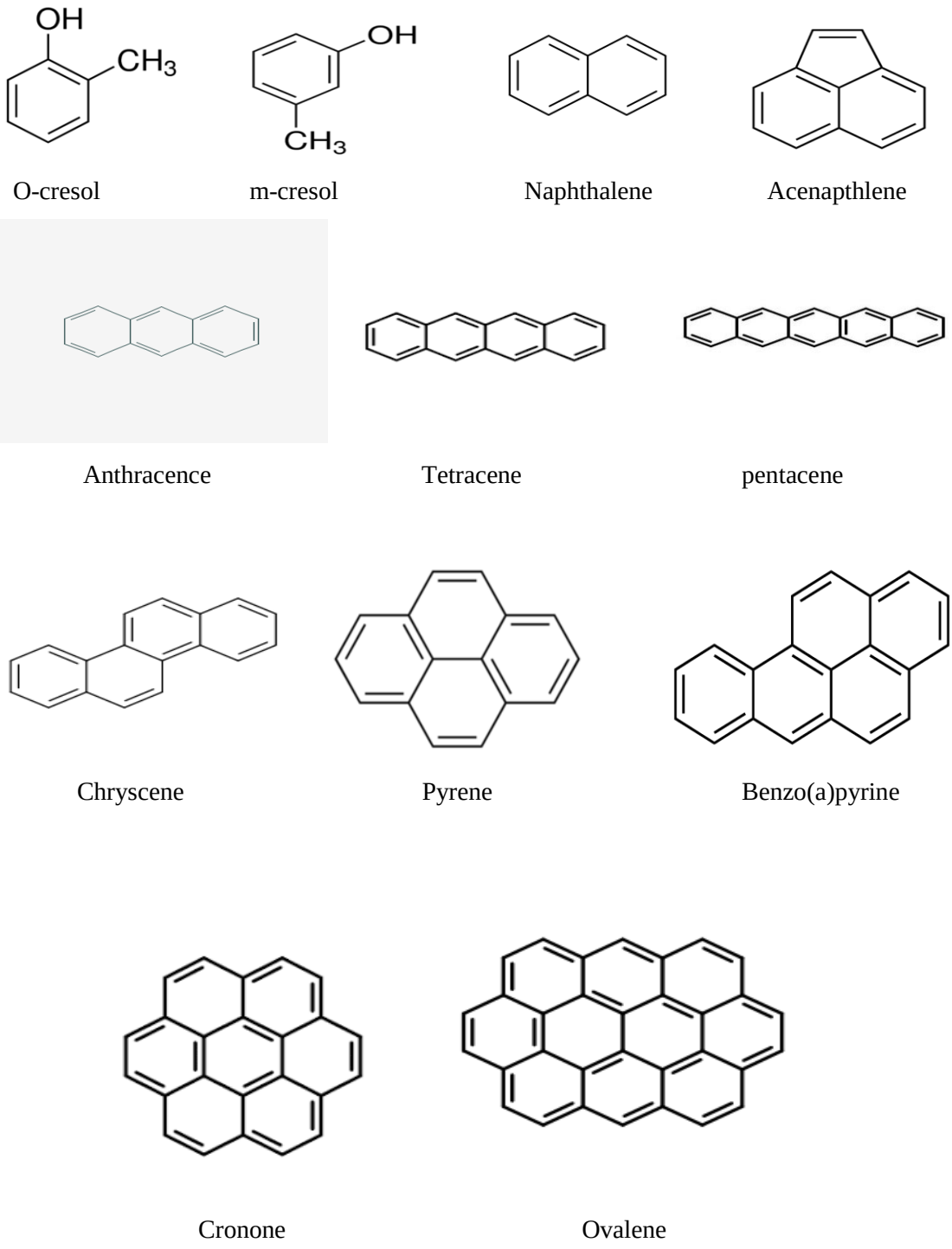


Figure 1: Chemical structure of phenol, other phenolic compounds and polyaromatic compounds commonly found in petroleum refinery effluents (Lide, 2004).

2.2. Phenol: A Major pollutant of wastewater

Phenol is one of the major constituent aromatic pollutants that are usually found in wastewaters from petroleum industries and oil refineries. Besides, phenol is also present in many other industrial effluents such as wastewaters from coal processing plants, pulp and paper manufacturing plants, resin and coke manufacturing, steel plants, pharmaceutical industries, plastic and varnish industries, textile units, pesticide plants, smelting and related metallurgical operations (Kumar et al., 2005; Edalatmanesh et al., 2008).

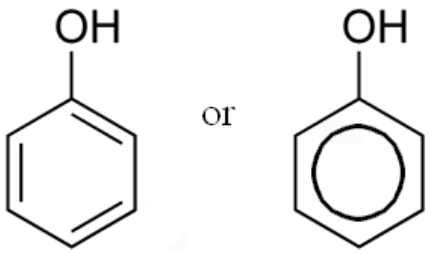


Figure 2: Structure of Phenol

Phenol, commonly known as carbolic acid, is a white crystalline solid having a strong odor and generally used in liquid form. It also known as hydroxybenzene, an aromatic compound having one hydroxyl group attached to the benzene ring. Phenol is the basic structural unit for a variety of synthetic organic compounds which is soluble in most organic solvent sand also fairly soluble in water with a solubility of about 83 g L−1 at 20 °C (ATSDR, 2008; EPA, 1979). It is moderately volatile at room temperature (evaporates more slowly than water) and quite flammable (Calabrase and Kenyon, 1991) and a sharp burning taste. Phenol is a weak acid and in its ionized form, it is very sensitive to electrophilic substitution reactions and oxidations. It is mildly acidic, but requires careful handling as it can cause burns. Phenols can be found either as natural or artificial mono-aromatic compounds in the environment as major pollutants. Phenol, in nature, can be generated from forest and rangeland fires and natural decay of lignocellulose material (Wang et al., 2007; Agarry et al., 2008). The chemical structure of phenol, other phenolic compounds and polyaromatic compounds commonly found in petroleum refinery effluents.

PROPERTY	PHENOL
Formula	C6H5OH
Molecular weight (g/mol)	94.14
Water solubility (g/L at 25 ºC)	87
Boiling point (ºC)	181.80

Melting point (ºC)	43
Flash point (open cup)	87
pka	9.89*10^-10
Auto ignition temperature	715 ºC

Table 1: Chemical and Physical properties of phenol

Natural and synthetic phenol can be produces with the help of chemical process. Phenol has been extracted from coal tar distillation naturally. Synthetically, cumene oxidation accounts for 95% of phenol production worldwide. The industries like leather, paint, pharmaceutical, coking plant petrochemical, oil refinery, plastic, explosives, steel, pesticides etc. and disinfectants uses phenolic and its derivative compounds as their products and raw materials leather, paint, pharmaceutical, paint ,coking plant petrochemical, steel, oil refinery, plastic, explosives, steel, pesticides etc. and disinfectants uses phenolic and its derivative compounds as their products and raw materials. (Busca et al., 2008).

2.3. Toxicity of Phenol:

Phenol is a major pollutant included in the list of EPA (1979) as reported by Agarry et al. (2008). Phenol is toxic even at low concentrations and the toxicity of phenols for microbial cells has been investigated (Keweloh et al., 1990; 1998; Kahru et al., 2002). Therefore, the environmentally unacceptable pollution effects of the wastes have been reported worldwide (Ruiz-Ordaz et al., 2001) and the adverse effects of phenol on health are well documented (Calabrase and Kenyon, 1991; Sax, 1984). Phenol and its derivatives (phenolics) are considered to be among the most recalcitrant and hazardous contaminants due to their high toxicity for human life, aquatic life and others (El-Naas et al., 2009; Shourian et al., 2009). Phenol and other phenolic compounds pose serious environmental threat as they can easily contaminate nearby water courses due to their water solubility and high toxicity (Mollaei et al., 2010).

Phenol can quickly penetrate the skin and may cause severe irritation to the eyes, mucous membranes and the respiratory tract. Hence, human exposure to phenol through ingestion, contact or inhalation may lead to critical health hazards and possible carcinogenesis (El-Naas et al., 2009). Furthermore, exposure to phenol by oral route may result in severe liver and kidney damage, and acute cardiac toxicity including weak pulse, cardiac depression, and reduced blood pressure. Ingestion of 1 g of phenol is reported to be lethal to human (Nuhoglu and Yalcin, 2005).

Phenol and other phenolic compounds have detrimental effects on the aquatic micro– flora and fauna at a very low concentration of 5 mg \L (Santos et al., 2009). Although, the concentration of phenol in wastewater generally varies from 10 mg \L to 300 mg \L, it can occasionally reach as high as 4.5 g\L in highly contaminated wastewater. Phenol may either exert its toxic effect by reducing enzyme activity or

even be lethal to fish at relatively low levels of 5–25 mg\ L, depending on the temperature and state of maturity of rainbow trout (Brown et al., 1967). Moreover, phenol contaminated wastewater, when chlorinated for disinfection, produces highly toxic polychlorinated phenols. Phenol, even at a very low concentration of 2 μg \L, imparts objectionable taste and odor to drinking water (Chung et al., 2003). Also, phenol and its homologues can adversely affect the performance of the wastewater treatment plants by inhibiting the microbial growth (Ren and Frymier, 2003).

Due to their potential toxicity, the United States Environmental Protection Agency (USEPA) has defined phenol and other phenolic compounds as priority pollutants and set a water purification level of phenol concentration less than 1 μg\L in surface waters (Keith and Telliand, 1979). The World Health Organization (WHO) has set their guideline of 1 μg\L to regulate the phenol concentration in drinking waters (Environmental Health Criteria-EHC 161, 1994). Also, a limit of 0.5 μg\L has been directed by the European Council Directive for regulating phenol concentration in the drinking waters (Tziotzios et al., 2005). Whereas, 0.35 mg\L is kept for effluent phenol concentration for petroleum oil refineries by Central Pollution Control Board, India (Ministry of Environment and Forest, Govt. of India).

2.4. Use of phenol:

As a pure substance, phenol finds its application in the production of slimicides, disinfectants, antiseptics and medicinal preparations such as ear and nose drops, mouthwashes and sore throat lozenges (ATSDR, 2008). Phenol is used for the preparation of some cream and shaving soap for its germicidal and local anesthetic properties, in veterinary medicine as an internal antiseptic and gastric anesthetic, as a peptizing agent in glue, as an extracting solvent in refinery and lubricant production, as a blocking agent for blocked isocyanate monomers, as a reagent in chemical analysis and as a primary petrochemical intermediate.

Industrially phenol is used for the production of phenolic resins like phenol– formaldehyde resins (Bakelite) which are low-cost thermosetting resins applied as plywood adhesive, construction, automotive and appliance industries. Even for the production of various epoxy resins phenol acts as the precursor compound. By reaction with acetone it may also be converted into bisphenol A, a monomer for epoxy-resins. It is also used to produce cyclohexanone and cyclohexanone cyclohexanol mixtures by selective catalytic hydrogenation. Cyclohexanone is later converted into its oxime and further to caprolactame, the monomer for nylon 6. Nitric acid oxidizes to adipic acid from the mixture of cyclohexanone cyclohexanol. Phenol is also used to produce polysulphone and polyphenoxy polymers, corrosion resistant polyester and polyester polyols.

Phenol is also a building block for the synthesis of pharmaceuticals, such as, e.g., aspirin (Busca et al., 2008). Phenol is used along with chloroform (a commonly-used mixture in molecular biology for DNA

& RNA purification from proteins) and also used for cell disruption and lysis purpose (Sambrook et al., 2000).

2.5 Phenol as disinfectant:

. Phenol is probably the oldest known disinfectant introduced by Lister as “carbolic acid”. Today disinfectants are widely used in the health care, food and pharmaceutical sectors to prevent unwanted microorganisms from causing disease. Phenolic disinfectants include O-Syl, Matar, Septicol, Hexachlorophene, Environ, One-Stroke, Lysovet, Tek-Trol, Lysol, Pantek, Discan, Pine-sol and Staphene. Phenol-based disinfectants such as Wex-cide, ProSpray, and Birex are germicidal, fungicidal, virucidal, and tuberculocidal i.e. these are effective against many bacteria, fungi and some viruses which are available in moderate cost (Thiel, 1999). Phenols are effective especially against gram positive bacteria and enveloped viruses such as Coronavirus BRS, BVD, IBR, PI3, Pox, Rabies Leukemia and Stomatitis virus. Phenolic compounds are used as intermediate level disinfectants used to treat non critical medical devices which pose the lowest risk of transmission of infection, usually contact only intact skin (Ritcher, 2011).They retain more activity in the presence of organic material than iodine or chlorine-containing disinfectants. These are commonly used in commercial animal production units including hatchery and equipment sanitation, and footbaths (Jeffrey, 1997). Phenolic disinfectants (including cresols and pine oil) are generally safe, but prolonged exposure to the skin may cause irritation.

2.6. Amount of phenol released in environment:

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

INDUSTRY	**PHENOL CONCENTRATION(mg\L)**
Coking operations	28 – 3900
Coal processing	9 – 6800
Petrochemicals	2.8 – 1220
Pulp and paper	0.1 – 1600
Gas production	4000

Refineries	6 – 500
Pharmaceuticals	1000
Benzene manufacturing	50

Table 2: Phenol concentration in industrial effluents (BUSCA ET AL. 2008)

Different industries releases different amount of phenol in the atmosphere. Some of the industrial survey is given in form of table below.

NAMES OF INDUSTRIES	**CONC. OF PHENOL RELEASED(mg/L)**
BILT paper industries	1400-1500
Barauni petroleum refinery	450-550
Paradeep petrochemicals	1000-1160
Rourkela steel plant	Up to 3500(for coaking process)

Table 3: Amount of phenol released by different industries

2.7. Treatment methods for the removal of phenolic waste:

In view of the wide prevalence of phenols in different wastewaters and their toxicity to human and animal life even at low concentrations, it is extremely necessary to remove them before discharge of wastewater into water bodies Therefore, it is very important to employ appropriate strategies of wastewater treatment in order to counterbalance these growing environmental problems. There are several treatment technologies have been employed in this regard (Klein and Lee, 1978; Anselmo and Novais, 1992; Koyama et al., 1994; Mokrini et al., 1997; Chan and Fu, 1998; Danis et al., 1998; Reardon et al., 2000; Backhaus et al., 2001; Goncharuk et al., 2002; Ajay et al., 2004). There are several ways to remove phenol from the industrial effluent or from the phenol contaminated water this methods are other than the biodegradation method for phenol. Various methods use for phenol separation are electro coagulation, extraction, adsorption and ion exchange, advanced oxidation process, electro-fenton method, membrane separation, photodecomposition etc. This all methods are used for effective phenol separation.

The applied treatment, which could be a single treatment or a combination of these treatments, must guarantee the removal of phenol to allowable discharge limits. The choice of treatment depends upon the concentration, and volume of the effluent treated and cost of the treatment.

2.8. Physico-chemical methods for removal of phenol:

A variety of treatment methods, such as adsorption, wet oxidation, chemical oxidation etc have been used for removal of phenols from aqueous solutions. Several treatment methods that are available for treating the phenolic waste include granular activated carbon processes and reverse osmosis, anaerobic processes, the electro Fenton method, and combined applications of flotation and coagulation processes, stripping and oxidation.

2.8.1 Ion exchange:

A variety of treatment methods, such as adsorption, wet oxidation, chemical oxidation etc have been used for removal of phenols from aqueous solutions. Several treatment methods that are available for treating the phenolic waste include granular activated carbon processes and reverse osmosis, anaerobic processes, the electro Fenton method, and combined applications of flotation and coagulation processes, stripping and oxidation.

2.8.2 Adsorption:

Adsorption is a widely used wastewater treatment method for color, heavy metals and other inorganic and organic impurities present in the industrial effluents as stated by Al-Rekabi et al., (2007) and Patel and Vashi. (2010). Adsorption of phenol onto activated carbon is a widely studied treatment method because of the affinity of phenols for the active surface of carbon (Garcia-Araya et al., 2003). Experiments were conducted to examine the liquid phase adsorption of phenol from contaminated eater using the silica gel, activated carbon and alumina by (Roostaei and Tezel Caetano et.al.) also carried out the same experiment of adsorption of phenol on the polymeric resins. Mechanisms involved in the adsorption process mainly focus on the selection of the adsorbent material like their particle size surface area and porosity etc. (Gardea-Torresdey et al., 2004). Kyuya et al. (2004) reported pore size distribution and surface area of the activated carbon are two important factors that affect the adsorption of phenol. Because of the high cost involved in the usage of activated carbon, materials basically obtained from low-cost agricultural wastes; activated carbon prepared from various raw materials such as sawdust, nut shells, coconut shells etc. Where been used as adsorbent (Zawani et al., 2009). These adsorbents are basically used for the effective removal and recovery of phenolic pollutants from wastewater streams (Basso et al., 2002; Park et al., 2006). Adsorption equilibrium capacities and kinetics analysis were carried out and it has found to be a promising

method for phenol removal. Phenol removal ranging from 88 % to 95 % was observed using various adsorbent. Adsorption seems to be possibly most widely studied operation for phenol.

2.8.3 Chemical oxidation:

Chemical treatment involves the use of chemical agents to completely destroy or convert the contaminants to harmless or less toxic compounds, or intermediates that can be further degraded by microorganisms (Hamby D. M., 1996). Abdelvahab et.al. have carried out the research on electrochemical removal of phenol from oil refinery waste Chemical oxidation of organic pollutants especially phenol is a promising alternative when wastewater contains no biodegradable and/or toxic contaminants and also when the contaminant concentration is high. In this process of chemical oxidation separation of phenol they used a tank with horizontal aluminum rods and the aluminium screen acting as anode. They studied the phenol removal with respect to various parameters such as pH, operating time, current density, initial phenol concentration and addition of NaCl. According to them removal of phenol during electro coagulation was due to combined effect of sweep coagulation and adsorption. The chemical agents used are normally strong oxidants. The most commonly used oxidants that initiate the oxidation reactions include hydrogen peroxide which is widely used for this purpose (Dias-Machado et al., 2006; Ksibi, 2006).The research studies showed that the 97% of separation was obtained after 2 hours of time at the operation pH of 7.

The chemical agents used are normally strong oxidants. Hydrogen peroxide is most commonly used oxidants that initials the oxidation reaction which is widely used for this purpose. (Dias-Machado et al., 2006; Ksibi, 2006).

2.8.4. Extraction:

Extraction is the most common process for separation of many compounds from each other. Extraction has been used in various industrial applications. Extraction is the process in which solvent is used to dissolve phenol from the effluent and then this solvent is separated by the distillation process. This process is very common but the important factor here is the selection of the solvent by the help of which we can separate the phenol. The solvent should be selected in such a way that it could absorb the maximum amount of the phenol and separation of phenol from the solvent should be economic and more viable. The solvent which is selected for the separation or the extraction process should be non-toxic in nature. There are many organic compounds which can be used for phenol as the solvents. 1-hexanol, 1-heptanol and 1 octanol can be used as solvent for phenol removal.

2.9. Limitation of the above wastewater treatment technique:

The physico-chemical treatment technologies discussed above found to have inherent drawback 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 requires further treatment and thus are alarming for the environment. By using these methods it is like trading one toxic element at the expense of other. The ion-exchange resins used in the adsorption process are too costly for the process to get fit into the economic agenda.

The main drawback with ion exchange method is the high cost of the ion exchange resins and each resin must be selectively removes one type of contaminant only. Caetano et al., 2009 reported that the phenol is removed by the ion exchange resins only in the alkaline medium while the maximum phenol removal was obtained by the non-functionalized resin in acidic medium. Moreover recovery of these ion exchange resins was tasking process. Further ion exchange is also highly sensitive to pH of the solution (Saparia et al., 1996; Liotta et al., 2009).

In adsorption, phenol in the wastewater is selectively transferred into the solid phase (adsorbent) instead of eliminating it from the wastewater. It once again produces a large amount of solid waste, which further requires a safe disposal. As mentioned earlier, use of activated carbon is not cost effective as high cost factors are associated with the recovery of activated carbon particles from the treated waste water (Banat et al., 2000).

The studies in various field of different methods for phenol degradation is appreciative, but among the above mentioned and discussed process none of the above process can be performed on the large scale. Moreover all the above method are pH sensitive and hence the maintenance of the accurate pH becomes the most difficult part to be handled where the large scale processes have been in use. Also the phenol which is being separated from all the above process is not processed further. The solid crystals of phenol are obtained and these are in form of waste and again needs a safe disposal. For example consider the extraction process. After separating phenol from the solvent it's again a solid waste which needs to be disposed of or been recycled to industries which use phenol for the production of various products. The various product industries producing cosmetics, antiseptics, etc. can import this waste phenol from the separation units.

There are many disadvantages associated with the chemical oxidation process like the high cost of the chemicals, emission of various harmful by products, it creates hazardous constituent like secondary effluent problem along with the production of harmful gases (Jena et al., 2005). In case of chemical oxidation of phenol, various oxidizing agents such as hydrogen peroxide, fenton's reagents etc. are used. Hydrogen peroxide when used alone has low reactivity and causes incomplete oxidation of many organic contaminants (Kamenev et al., 1995; Ikehata and Gamal El-Din, 2006).

Hence, detoxification and degradation of phenol without the above mentioned drawbacks has become the focus of the research the development of technology. Biological treatment with pure and mixed microbial strains is considered to be an attractive and efficient alternative for the treatment of contaminated wastewaters containing recalcitrant substances such as phenolics since it produces no toxic end products and it is cost effective (Monteiro et al., 2000; Banerjee et al., 2001; Abuhamed et al., 2004; Kumar et al., 2005; Rodriguez et al., 2006).

2.10. Biodegradation of Phenol:

Biodegradation is the breakdown of complex and possibly toxic organic contaminants to non-toxic like water, carbon dioxide and biomass by microbial activity such as bacteria, fungi and algae. These contaminants can be considered as the microbial food source or substrate. Biodegradation of any organic compound can be thought of as a series of biological degradation steps or a pathway that ultimately results in the oxidation of the parent compound that often results in the generation of energy. Microorganisms have the capability of degrading all naturally occurring compounds; this is known as the principle of microbial infallibility (Alexander, 1965). However, biodegradation is limited in the number of toxic materials it can handle, but where applicable, it is cost effective (Atlas and Unterman, 1999).The biodegradation method of phenol removal draws universal preference due to the possibility of complete mineralization of phenol (El-Naas et al., 2009; Liu et al., 2009), avoiding undesirable by-products or secondary pollutants produced as a result of physico-chemical treatment of phenol containing wastewater.

2.11. Advantages of biodegradation:

There are several advantages of biodegradation over all the above methods which were discussed for removal of phenol from the toxic effluent. First advantage of microbial degradation is the pathway that the microorganisms follow which is capable of breakage of these toxic compounds into the simpler and nontoxic compounds. It produces no harmful end products, cost effective 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. Also this method does not involve usage of costly commercial equipment such as the distillation column or high temperature reactors hence it is economical for both small scale and large scale operations. This method also eliminates the cost of the costly chemicals used for oxidation reaction and risk factor of handling those reactive oxidizing agent. At last this process can be done at room temperatures as microorganisms growing conditions are at room temperature itself. Alexander in 1995 have explained that microorganism have the capability of degrading any compound. This capability of microorganism is called microbial infallibility. But a single microorganism cannot

degrade or survive in all kind of conditions. Specific microorganism can only degrade a specific compound or in other words we can say that specific microorganism take only specific compounds as their food.

The only problem regarding the use of this method is that it needs to be optimized and microorganism or biomass used for this method should be checked because if the dead mass increases in the reacting vessel it can clog the flow pattern of the input and output streams.

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2.12. Mechanism of biodegradation of phenol:

2.12.1. Aerobic biodegradation: In microbial degradation of phenol under aerobic conditions, the degradation is initiated by oxygenation in which the aromatic ring is initially monohydroxylated by a mono oxygenase phenol hydroxylase at a position ortho to the pre-existing hydroxyl group to form catechol. This is the main intermediate resulting from metabolism of phenol by different microbial strains.

Depending on the type of strain, the catechol then undergoes a ring cleavage that can occur either at the ortho position thus initiating the ortho pathway that leads to the formation of succinyl Co-A and acetyl Co-A or at the meta position thus initiating the meta pathway that leads to the formation of pyruvate and acetaldehyde. Hill and Robinson (1975), Ghadi and Sangodkar, (1995) have described the biodegradation or metabolism of phenol via the meta cleavage pathway, while Gaal and Neujahr (1980) and Paller et al. (1995) described the biodegradation of phenol by *Trichosporon cutaneum* and *Acinetobacter calcoaceticus* respectively via the ortho cleavage pathway.

META-PATHWAY FOR PHENOL DEGRADATION:

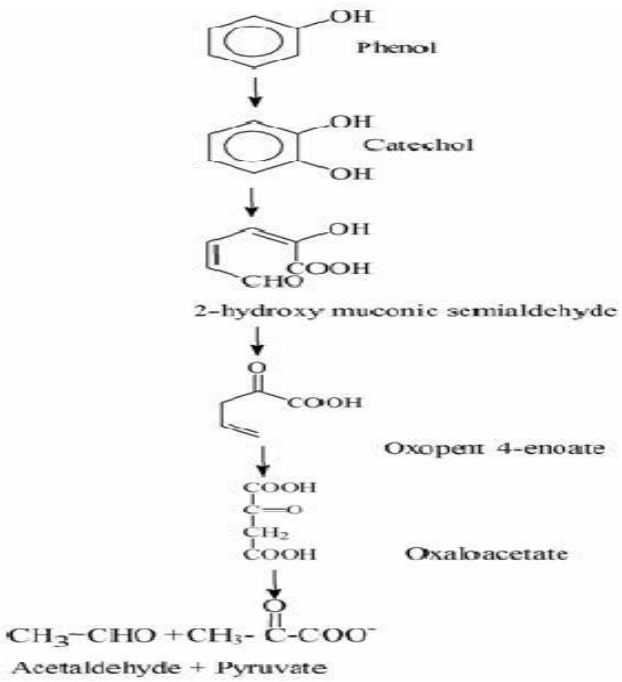


Figure 3: Meta pathway for phenol degradation

ORTHO-PATHWAY FOR PHENOL DEGRADATION:

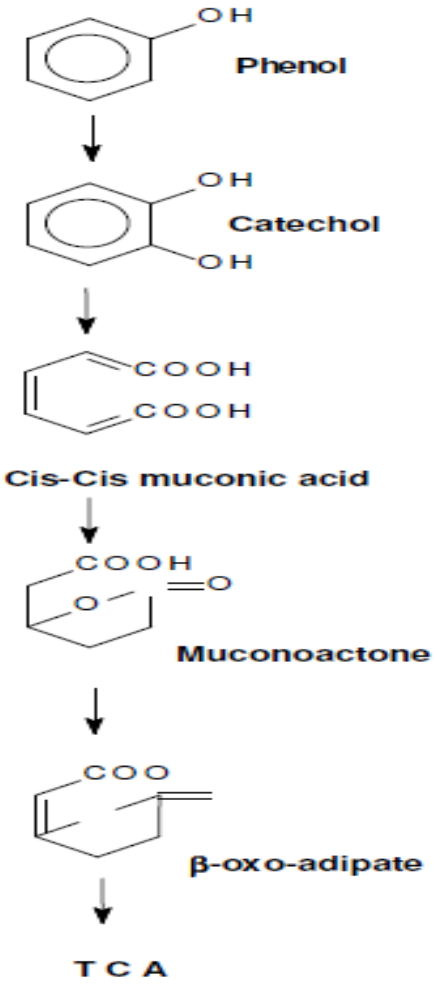


Fig 4: Ortho-pathway for phenol degradation

2.12.2. Anaerobic biodegradation:

The microbial degradation of phenol under anaerobic conditions has been studied in the denitrifying bacterium *Thauera aromatica* (Aresta et al., 1998; Lack and Fuchs, 1992, 1994). The anaerobic degradation is initiated via carboxylation of phenol. The phenol carboxylation proceeds in two steps. The first step involves the phosphorylation of the phenol by the addition of a phosphate group from an unknown phosphoryl donor catalyzed by a phosphorylated enzyme called phenyl phosphate synthase (kinase) to form phenyl phosphate as the first intermediate (Lack and Fuchs, 1992, 1994). The second step involves the carboxylation of phenyl phosphate catalysed by a Mn2+ requiring enzyme, phenyl phosphate carboxylase to form 4-hydroxybenzoate. The synthesis of both the phosphorylating and carboxylating enzymes is strictly regulated. The phenol carboxylating enzyme in *Thauera aromatica* does not belong to any group of the studied carboxylases as it seems to proceed via a phosphorylated free intermediate. It is extremely

oxygen sensitive and sensitive to radical trapping agents, it is not dependent on biotin or thiamine diphosphate and differs from most known carboxylases by using carbon dioxide as substrate and a metal as co-catalyst (Breining et al., 2000). from both the above mechanism we come to know about the different pathways followed by which the microorganisms degrade phenol and their last products. In Meta pathway its acetaldehyde and pyruvate and in Ortho pathway its TCA (tri-carboxylic acid).

2.13. Phenol degrading microorganism:

The hazardous and toxic xenobiotics, being structurally different from naturally occurring compounds, are difficult to degrade. However, due to rapid advancement in the field of biotechnological research in recent years, a wide range of microorganisms have been identified which can utilize toxic xenobiotics to meet their metabolic requirement. In this regard, a large number of microorganisms including bacteria, fungi and algae have been demonstrated to degrade phenol and other phenolic compounds. Phenol can be degraded either aerobically (Ruiz-Ordaz et al., 2001; Melo et al., 2005; Ucun et al., 2010) or anaerobically (Levénet al., 2006; Azbar et al., 2009; Bajaj et al., 2009; Hussain et al., 2010), depending on the specific growth conditions required by the microorganisms.

The focus on the microbial degradation of phenols in recent years has resulted in the isolation, culture, adaptation and enrichment of a number of microorganisms that can grow on the compound as a sole carbon and energy source. Phenol is an antimicrobial agent; many of the microbes are susceptible to this compound. However, there are some microbes, which are resistant to phenol and have the ability to degrade phenol.

2.13.1. Bacteria:-

In recent years, the biodegradation of phenol and other phenolic compounds by bacteria has been the subject of extensive research in the field of biochemical as well as environmental engineering. This has led to the isolation and characterization, at both the physiological and genetic level, of a wide variety of bacteria that are capable of phenol biodegradation. Undoubtedly, *Pseudomonas putida* and other members of *Pseudomonas* genus are the most widely investigated bacteria in this regard due to their high phenol removal efficiency (Hsieh et al., 2008; El-Naas et al., 2010). Several which have been studied for phenol biodegradation include *Acinetubacter sp.*, *Sphingomonas sp.*, *Ewingella sp.*, *Alcaligenes sp.*, *Ochrobactrum sp.*, *Ralstonia sp.*, and *Bacillus sp.*

Amongst all the microorganisms listed above genus Pseudomonas comprises an important group of bacteria with environmental application in bioremediation and biological control (Agarry et al., 2008). In Pseudomonads, many of its induced enzymes are nonspecific and its metabolic pathway contains a high

degree of convergence. The convergence of catabolic pathways allow for the efficient utilization of a wide range of growth substrates while the non-specificity of the induced enzymes allows for the simultaneous utilization of several similar substrates without an excess of redundant genetic coding for enzyme induction (Hannaford and Kuek, 1999; Monteiro et al., 2000; Banerjee et al., 2001; Abhuhamed et al., 2004; Kumar et al., 2005; Nuhoglu and Yalcin, 2005; Karigar et al., 2006; Rodriguez et al., 2006).

Moreover, some yeast such as *Candida tropicalis* (Ehrhardt and Rehm, 1985), *Fusarium flocciferium* (Anselmo et al., 1985; Mendoca et al., 2004) and *Trichosporoncutaneum* (Gaal and Neujahr, 1980; Yang and Humphrey, 1975) are also capable of degrading phenol.

Microorganisms are able to adapt to the presence of toxic organic compounds by using a whole cascade of adaptive mechanisms. Among the adaptive mechanisms, changes in the fatty acid composition of membrane lipids are the most important reactions of bacteria to membrane-active substances (Neumann et al; 2004).

Kotturi et al. (1991) have studied cell growth and phenol degradation kinetics at 10°C for a psychrotrophic bacterium, *Pseudomonas putida* Q5. The batch studies were conducted for initial phenol concentrations, ranging from 14 to 1000mg/1. The experimental data were fitted by non-linear regression to the integrated Haldane substrate inhibition growth rate model. The values of the kinetic parameters were determined. Compared to mesophilic pseudomonads previously studied, the psychrotrophic strain grows on and degrades phenol at rates that are ca. 65-80% lower. However, use of the psychrotrophic microorganism may still be economically advantageous for waste-water treatment processes installed in cold climatic regions, and in cases where influent waste-water temperatures exhibit seasonal variation in the range 10-30 ° C.

2.13.2. Fungi:-

Fungi are stout microorganisms in a sense that they can tolerate the presence of high concentration of various toxic materials, even at a low bioavailability exploiting their powerful extracellular oxidative enzymatic system (Agarry et al., 2008). Fungi also play their part in the biodegradation of phenol. Yeasts are the commonly found fungi in the contaminated environments and some of them such as *Candida tropicalis*, *Fusarium flocciferium* and *Trichosporon cutaneum* are capable of utilizing phenol as the major carbon and energy source (Stoilova et al., 2007; Agarry et al., 2008). Also, strains from *Penicillium*, *Aspergillus*, *Graphium* and *Phanerochate* genera have been reported to disintegrate phenol and other aromatic pollutants.

Thirty filamentous fungal strains) isolated from effluents of a stainless steel industry (Minas Gerais, Brazil) and tested for phenol tolerance. Fifteen strains of the genera *Fusarium* sp., *Aspergillus* sp., *Penicillium* sp. and *Graphium* sp. tolerants up to 10 mM of phenol were selected and tested for their ability

to degrade phenol. Phenol degradation was a function of strain, time of incubation and initial phenol concentration. FIB4, LEA5 and AE2 strains of *Graphium* sp. and FE11 of *Fusarium* sp. presented the highest percentage phenol degradation, with 75% degradation of 10mM phenol in 168 hours for FIB4. A higher starting cell density of *Graphium* sp. FIB4 lead to a decrease in the time needed for full phenol degradation and increased the phenol degradation rate. All strains exhibited activity of catechol 1,2-dioxygenase and phenol hydroxylase in free cell extracts obtained from cells grown on phenol, suggesting that catechol was oxidized by the *ortho* type of ring fission.

2.13.3. Algae:-

Recently, much interest is being drawn to the investigation of degrading phenol by using algae. Although most of the algae have low tolerance to the acute toxicity of phenols, some cyanobacteria and eukaryotic microalge such as *Chlorellasp. Scenedesmus sp., Selenastrum capricornutum, Tetraselmis marina, Nostocpunctiforme and Oscillatoria animalis* have been reported to be capable of bio transforming phenolic compounds (Lika and Papadakis, 2009).

2.14. Enzyme responsible for the biodegradation:

There are reports on many microorganisms capable of degrading phenol through the action of variety of enzymes. These enzymes may include oxygenase hydroxylases, peroxidases, tyrosinases and oxidases.

2.14.1. Oxogenasis:

It is the enzyme that changes the hydrophobic organic compound to more water-soluble and thus it can be broken down by a larger number of other microorganisms. Two major classes of oxygenases are known. They are monooxygenase and dioxygenase. These enzymes participate in the oxidative metabolism of a wide variety of chemicals of agricultural pharmaceutical, and environmental significance. Aliphatic and aromatic hydrocarbons are most widely recognized substrates for this class of enzymes for both of endobiotic and xenobiotic sources.

2.14.2 Monooxygenases.

This class of enzymes inserts one atom of the oxygen molecule into the substrate, and the other atom of oxygen becomes reduced to water, i.e. two reluctant (substrates) are needed. They are also more complex in action, and can catalyze several different types of O insertion reactions. Mnoxygenases oxidize 2 substrates therefore they are also called mixed function oxidizes and one of the main substrates that becomes hydroxylated are called *h*ydroxylases.

The general stoichiometry is as follows:

$$R\text{-}H + NAD(P)H + O_2 \text{-----} > R\text{-}OH + NAD(P) + H_2O$$

2.14.3 Dioxygenases:-

Dioxygenases incorporate both atoms of the oxygen molecule into the substrates. Chlorinated and nitro-aromatic compounds as well as non-substituted PAHs are very important in initiating the biodegradation of these Dioxygenases. These compounds are first degraded to catechol or protocatechuate by oxygenases (both dioxygenases and monooxygenases). The intermediates are metabolized by ring-cleavage type of dioxygenases to either beta-ketoadipate or 2 keto-4-hydroxyvalerate. These intermediates then enter the TCA cycle.

2.14.4 Hydoxylase*:*

Phenol hydroxylase catalyses the degradation of phenol via two different pathways initiated either by ortho or meta cleavage. Biodegradation of phenol involves hydroxylase and catechol 2, 3 dioxygenase are been reported. (Leonard and Lindley, 1998). Phenol-degrading aerobic bacteria are able to convert phenol into nontoxic intermediates of the tricarboxylic acid cycle via an ortho or meta pathway (Harwood and Parales, 1996).. The monooxygenation of the aromatic ring constitutes the first step in the biodegradation of many phenolic compounds. This process is carried out by flavoprotein monooxygenases, which use electrons of NAD(P)H to activate and cleave a molecule of oxygen through the formation of an intermediate flavin hydroperoxide and enable the incorporation of an oxygen atom into the substrate (Moonen et al., 2002).A single polypeptide chain or by multicomponent enzymes catalyzes the reaction (van Berkel et al., 2006).A class of monooxygenases, consisting of a small reductase component that uses NAD(P)H to reduce a flavin that diffuses to a large oxygenase component that catalyzes the hydroxylation of aromatic substrate is reported. (van Berkel et al., 2006).

2.15. Parameters affecting microbial degradation of phenols:

Various biotic and abiotic parameters such as substrate concentration, temperature, pH, oxygen content, bioavailability and physico-chemical properties of the pollutant can influence the multifaceted process of phenol biodegradation by either enhancing or retarding the microbial metabolism (Agarry et al., 2008; El-Naas et al., 2009; Trigo et al., 2009). Therefore, in order to achieve the maximum phenol degradation, each of these parameters needs to be optimized for a specific microorganism.

2.15.1 pH:

The pH of the culture medium is an important parameter in phenol biodegradation as the extreme pH values (≤ 3 or ≥ 9) can exert detrimental effect on the microbial growth. Bandyopadhyay et al. (1998) studied the phenol biodegradation by *Pseudomonasputida* MTCC 1194 with varying medium pH ranging from pH 4 to pH 9; and demonstrated that the highest phenol degradation occurred at the neutral condition of pH 7. In their study of phenol degradation by free and immobilized *Acinetobacter sp.* strain PD12, Wang et al. (2007) showed that the degradation rate constant was almost invariable in the pH range of 7.2–10 for both the free and immobilized cells. However, it started to decrease below pH 7.2 for both free and immobilized system, ultimately resulting in the complete inhibition of the degrading activity at pH 5.5 in case of free cells. The effect of media pH on phenol degradation by *Ochrobactrum sp.* was studied by Kilic (2009) and the highest phenol degradation was observed at pH 8 in the investigated pH range of 6–9. Liu et al. (2009) investigated the degradation of phenol by mixed culture of *Acinetobacter sp.* XA05 and *Sphingomonas sp.* FG03; and found that the media pH did not have any significant effect on the phenol degradation, by free as well as immobilized cells, in the range of pH 7 – pH 9. However, the degradation by free cells gradually decreased in the acidic pH and reached almost complete inhibition at pH 6. On the other hand, the immobilized cells followed the same trend but still maintained an acceptable degradation rate at the same pH of 6.

2.15.2. Temperature:

Change in temperature can influence the process of phenol biodegradation as different microorganisms have different temperature range for their optimal growth. Although most of the phenol biodegradation studies had been performed in the temperature range 25–35 °C, successful phenol degradation has been reported at temperatures as low as 10 °C and as high as 50 °C by *Pseudomonas putida* and *Bacillusstearothermo-philes*, respectively (Agarry et al., 2008). The effect of temperature on the phenol degradation by *Pseudomonas sp.* SA01 was investigated by Shourian et al. (2009) at three different temperatures of 25 °C, 30 °C and 37 °C. They found that the maximum phenol biodegradation occurred at 30 °C. Liu et al. (2009) also showed that the mixed culture of *Acinetobacter sp.* XA05 and *Sphingomonas sp.* FG03 degraded maximum phenol at 30 °C. In this regard, El-Naas et al. (2009) proposed that exposure to temperatures higher than 35 °C could affect the bacterial enzymes responsible for the benzene ring cleavage, the critical step in the biodegradation of phenols.

2.15.3. Substrate Concentration:

The substrate concentration is an important parameter in the phenol biodegradation as phenol itself is well known to inhibit microbial growth, especially at higher concentrations. This phenomenon is

commonly known as 'substrate inhibition'. Adjei and Ohta (2000) reported that phenol was completely inhibitory for cyanide utilization by the bacteria *Burkholderia cepacia* strain C-3. *Bacillus brevis* was shown to degrade phenol completely at an initial phenol concentration as high as 1750 mg\l, although it took about 120 h as compared to that of 24 h and 48 h for 750 and 1000 mg\L phenol, respectively (Arutchelvan et al., 2006). In their study of phenol biodegradation by *Rhizobium sp.* CCNWTB 701 isolated from mining tailing region, Wei et al. (2008) investigated the effect of substrate concentration on the biodegradation efficiency by varying initial phenol concentration from 100 mg\ L to 1000 mg\ L. It was observed that the time for phenol degradation increased in high phenol concentration with ~99.5% and ~78.3% phenol degradation within 62 and 66 h, starting with an initial concentration of 900 and 1000 mg\ L, respectively. Also, the specific phenol degradation rate first increased and then decreased with increasing phenol concentration, while the maximum value was found at 400 mg\l. Similar results were also reported for the phenol degradation by *Pseudomonas sp.* SA01 (Shourian et al., 2009) where degradation time increased from 20 h to 85 h with increasing concentration of initial phenol concentration from 500 to 1000 mg \L, while complete inhibition of degradation capabilities was observed at 1200 mg\L.

2.15.4. Culture System:

The biodegradation of phe**n**ol can be carried out either by suspended microbial culture or immobilized microbial cells. It has been observed that immobilization of microbial cells onto solid supports such as alginate, poly-acrylamide or chitosan particles, diatomaceous earth, activated carbon, sintered glass, polyvinyl alcohol and polymeric membrane could result in enhanced degradation efficiency as compared to that of the free (suspension) cell system (El-Naas et al., 2009; Liu et al., 2009; Shourian et al., 2009). Moreover, the systems with immobilized cultures are more stable to shock loading than the suspended cultures with free cells (Gerrard et al., 2006). Kim et al. (2006) showed that calcium alginate immobilization of microbial cells effectively increased the tolerance of *Pseudomonas putida* MK1 to phenol and improved the degradation of pyridine in a binary mixture of the two compounds. In a study of phenol degradation by *Pseudomonas putida* entrapped within the beads prepared by chitosan cross-linked with sodium tri-polyphosphate, Hsieh et al. (2008) demonstrated that the entrapped cells were superior to the free suspended ones in terms of tolerance to the environmental loadings and repeating usage. Also, the entrapped cells degraded influent phenol completely in the packed column mode of operation with the addition of hydrogen peroxide in order to increase the dissolved oxygen level for cell metabolism.

2.15.5. Chemical Structure of Phenol:

The chemical structure of a particular pollutant determines, to a significant extent, the biodegradability and hence, the toxicity of that compound. The difference in chemical structure can be

substantiated by the degree of branching and the number, type or position of the substituents. As the number of substituents in the structure increases, in most cases, the degradability deceases and the toxicity of the pollutant increases. For example, substituted phenols such as mono, di-, tri- and penta- chlorophenol are less degradable than unsubstituted phenol. In addition, *ortho*- and *para* substituted phenol are more degradable than *meta* substituted phenols (Agarry et al., 2008).

2.15.6. Other Factors:

The presence of naturally occurring carbon sources such as glucose, yeast extract, etc. can greatly affect the efficiency of microbial degradation of toxic pollutants. In this regard, the co-metabolism process, which can be described as the degradation of a compound only in the presence of another organic material that serves as the primary growth substrate, is an ideal example of the substrate interaction during the biodegradation of pollutants (Annadurai et al., 2008). Co-metabolism has been attributed to the production of broad-specificity enzymes, where both the primary substrate and the other compound compete for the same enzyme (Bhatt et al., 2007). The biodegradation of phenol and phenolic compounds has been reported to increase with increasing concentrations of inorganic nutrients (such as phosphate and nitrate), whereas high concentrations of organic nutrients (such as glucose, yeast extract, etc.) could affect it adversely (Gladyshev et al. 1998). Also, the presence of metabolic inhibitors or competing substrates has significant impact on the bio-degradation system (Mordocco et al., 1999; Tsai and Juang, 2006).

2.16. Problem associated with plan of work:

From the above study it can be concluded that phenol is poisonous and dangerous substances even at very low concentration and it has to be removed or minimized present in the waste water to satisfactory level. As there are several chemical methods present for the accessible for handling of phenol but the biological handling is particularly attractive as it has likely to approximately involve in the degradation of phenol entirely by producing harmless last yield and least derivative dissipate production. Again, chemical process biodegradation are very costly and non-economic to be performed on large scale hence biodegradation of phenol is most economic process to degrade phenol from water then the biological process. The only drawback of biological process is that it is time consuming but if it works properly phenol can be removed from water most efficiently and economically while removal of phenol by chemical process like adsorption solvent extraction, incineration chemical oxidation, and other non-biological treatment methods suffer from serious drawbacks such as high cost and formation of hazardous by-products.

On the basis of phenol degrading capacity bacteria and fungi is been tested and calculated form experiment result. But all the above experiments the microorganisms are selected from the first and then their phenol degrading capacity is calculated. In our experiment we check the capabilities of marine

microorganism's different source of waste water. And selected those microorganisms that can be degrade the phenol to a large extend.

The work plan is explained in below steps:

- To find specific colonies degrading phenol by increasing the concentration of phenol.
- Degradation of phenol has to be measured and efficiency of different microorganism to degrade and time taken to reduce the concentration of phenol has to be measured.
- Effects of various parameter on phenol degradation:

    - pH
    - Temperature
    - Inoculum volume
    - Agitation speed

Chapter 3.

Aims and Objectives

The aim of this study is to optimize the bioremediation process parameters using microorganisms in batch scale study. Objective of the study is as follow:

- Isolation, screening and selection of microorganisms, which efficiently degrade phenol.
- Batch Scale studies on the culture growth and biodegradation of phenol by the isolate.
- Identification and characterization of unknown microorganism using biochemical test.
- Batch scale studies on the culture growth and biodegradation of phenol using some unknown microbes which are recently isolated.
- Optimizing process parameter by varying parameters such as pH, concentration, inoculum size, temperature etc.
- Growth study and variation due to effect of phenol concentration in the batch scale study.

Chapter: 4.

Materials and methods

4.1 Fungal biodegradation:

4.1.1 Required chemicals and culture media:

Phenol and chemicals, used in the study, were of analytical grade; glucose and inorganic salts, used in preparing microbial growth media, were of reagent grade. All the chemicals and reagents were procured HIMEDIA. Phenol(99% Assay) was used for the biodegradation study along with a basic mineral salt medium (MSM)containing 1.00 g\L Ammonium sulphate $(NH_4)_2SO_4$,1.00 g\L Potassium dihydrogen phosphate (KH_2PO_4), 0.5 g\L Magnesium sulfate ($MgSO_4.7H_2O$), 1.0 mg\L ferrous sulfate ($FeSO_4.5H_2O$), 50 μg\L copper sulfate ($CuSO_4.5H_2O$), 0.05 g\L calcium chloride ($CaCl_2.2H_2O$) was prepared. Solid media was prepared by the addition of 35.01 g\L Czapek Dox Broth, Granulated to the 20 g\L (2%) agar to the medium for the culturing. Prior to use, the media were sterilized in an autoclave at 121 °C for 15 min. The growth media were prepared by adding phenol of required concentration (50-500 mg\L) to the MSM. Initially for isolation process, the pH of the medium was maintained at 5.5 and the working volume was 250 mL in all experiments.

4.1.2. Isolation and preparation of subculture by enrichment method:

For the study, Alternaria alternata (F-1) and Aspergillus niger (F-9) fungi collected from Sunderban area was used which was previously isolated. These isolated fungi was subcultured for the further study. Subcultures are important to prepare because we store our microorganism in form of this pure cultures. Whatever the inoculum is needed for the degradation of the phenol is taken from these pure cultures. These pure cultures needed to be stored in very safe and dry place at 4°C temperature away from any impurities and contact of any other foreign microorganism. If it comes in contact of any foreign organism or in other words if the culture is disturbed by growth of any other microorganism in it, it will be very hard to know the impurity and the metabolism of actual microorganism will be disturbed hence carefully handling of microorganisms' pure cultures is needed after preparation of it. If the cultures get disturbed again the microorganisms needed to be isolated from the beginning.

The procedure/method for the preparation of sub culture (100ml) is:

- Measure 100ml distilled water with the help of measuring cylinder and take it in a 250 ml flat bottom conical flask.

- Measure 3.5g Czapek Dox Broth and 2.00g agar which will be added in 100ml water for preparing the media for growth of microorganisms.
- Dissolve Czapek Dox agar in water and sterilize it for about 15 minutes at a temperature of 121°C in autoclave for removal of any contamination if present.
- Pour the Czapek Dox agar in petriplates after letting it cool down to room temperature in laminar flow chamber.
- After media solidification, streak the media using pore culture and incubated it at 35°C for 3 days.

4.1.3 Screening of phenol tolerating strain:

For the screening, isolated fungi was made to grow on minimal salt media (MSM) agar plates at different phenol concentration ranging from 50 mg\L to 500 mg\L as sole source of carbon and incubated at 35°C for 3 days. After incubation period growth is observed at different phenol concentration. Microorganism utilized the phenol as their carbon source and differential growth is observed at different phenol concentration.

4.1.4 Preparation phenol stock solution:

For preparing stock solution of phenol at any concentration we have the phenol of 99-100% purity grade. Phenol of 100% concentration had been prepared as stock solution. Stock solution is very useful to prepare phenol solution at very low concentration. For example, if we want to prepare it from the 100% concentrated phenol in 100ml of solution, a minute amount needs very costly calibrated pipettes but most importantly it needs a high degree of accuracy in handling chemical. Hence if stock solution of say 1000 ppm is prepare and kept the low concentration solutions can be prepared easily.

There is calculation involved in this procedure,

Let ‘x’ be the concentration of the stock solution needed to prepare in ppm. Let ‘y’ be the volume of the pure culture needed to prepare 100ml stock solution of concentration ‘x’.Hence, from balancing we know that for molar balance to be equal we have,

$$M1*V1 = M2*V2$$

$$x*100 = 106*y$$

$$y=10^4/x$$

4.1.5 Preparation of inoculum and spore collection:

4.1.5.1. Inoculation preparation of fungal spores:

- Prepare a spore suspension by pouring 5–7 mL of sterile 0.9 % NaCl (w/v) + 0.01 % Tween 80 on a Czapek Dox Agar (CDA) plates containing a premade fungal culture, and scrape the spores into solution.
- Filter the spore solution into a sterile 10 mL vial tube through a sterile funnel containing a cotton wool plug to remove hyphae and centrifuge it to 1000 rpm and discard the solution
- Repeat the previous process 2 to 3 times to remove hyphae with dilution of autoclaved distilled water.
- Take 1 mL of the spore solution and dilute further if required for obtaining a viable count or separate colonies, using aseptic techniques. Plate out 100 µL aliquots and spread aseptically using a sterile spreader. A glass spreader can be sterilized by flaming in 70 % (v/v) ethanol.

4.1.5.2 Method for counting fungal spores using haemocytometer:

Preparation of Neubauer's haemocytometer:

- Clean all surfaces of the haemocytometer and cover glass, take care to ensure that all surfaces are completely dry using lint-free tissue, center the coverslip on the hemacytometer.

- Put 9µl of the cell suspension into one of two counting chambers of Neubauer's haemocytometer. Use a clean pipette tip. Be sure that the suspension is thoroughly, but gently, mixed before drawing the samples. Fill the chambers slowly and steadily. Avoid injecting bubbles into the chambers. Do not overfill or under-fill the chamber.

- Count the fungal spores under light microscope. Don't count talc particles of the product in the suspension. Count all of the fungal spores in four corner squares of the chamber. Calculate the total number of the fungal spores counted in 4 squares. Calculate concentration of fungal spores in the suspension using the equation: spores/ml = n x 10^4, where n is the average number of spores counted in 4 squares of the chamber. Don't count the spores touching the top or left borders and don't count the spores touching the bottom or right borders of squares.

- Recalculate the concentration of the fungal spores in the product according dilution of suspension.

4.1.6 Phenol biodegradation studies:

For degradation of phenol we need to prepare the minimal salt media. We can wary the initial concentration of phenol by the spectrophotometric method and absorbance was checked. Minimal salt media is prepared instead of the nutrient broth so that all the nutrients that the organisms are getting from the nutrient broth may be stopped like carbon source or nitrogen source and we can add phenol externally as carbon source. According to the phenol concentration of solution of 100 ml needed to prepare the amount of stock solution is taken and the rest distilled water is added to make over up to 100 ml. The minimal salt media provides the conditions of minimal salt required of the growth of the microorganisms. It provides the necessary nitrogen source and the metals in trace amount like calcium and iron.

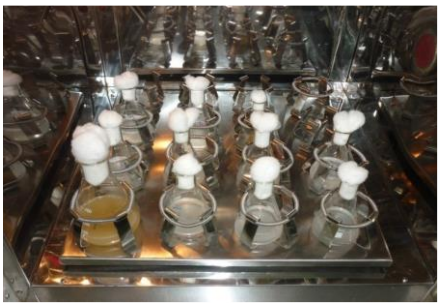


Figure 5: Microbial growth and Degradation in incubator

Figure 6: syringe filter (Sartorius NY 0.2 µL)

All biodegradation experiments were performed in 250 ml Erlenmeyer flask containing 100 ml of MSM containing phenol at concentration ranging from 100 mg/L to 250 mg/L. Upon incubation of the flasks at 30°C under agitation condition (150 rpm), samples were withdrawn at regular time interval and filtered using syringe nano filter such that no residue is present other than phenol. After the filtration each samples were analyzed using spectrophotometer at 268 nm.

4.1.7 Batch scale study for optimization of process parameter:

4.1.7.1 Degradation of phenol at different temperature and different pH:

Degradation of phenol was studied at different temperatures below 50°C because all the naturally existing microorganisms in nature lose their growth at such a high temperature. Three different temperature 25°C, 35°C and 45°C was studied to detect optimal growth of the microorganism. Also for pH the different pH ranging from pH 2 to pH 8 were used to detect the growth of microorganisms and the best one was detected with the maximum growth. The steps for the procedure is as follow:

- Prepare minimal salt media (MSM) in 2 liter of Erlenmeyer conical flask and Measure 100ml MSM with the help of measuring cylinder and take it in a 250 ml flat bottom conical flask and autoclaved it to 121°C for 15 minutes.
- Adjust pH of the each conical using buffers (acid & base) as per requirement for the experiment.
- Inoculate phenol for each flask by diluting from the phenol stock solution as per the requirement and check pH each of the conical.
- Inoculate 10µl of fungal spore to the each of the conical for the degradation for the study and incubate it at 130 rpm and different temperature for the study.
- Take sample from each of the conical for regular interval (24 hrs.) of time and measure absorbance by spectroscopic method for temperature and pH study.

4.1.7.2 Degradation of phenol at different phenol concentration and inoculum volume:

Till now, the inoculation was done with the constant phenol concentration inoculum of fungal spores at constant volume. But the degradation of phenol can be checked by inoculating the media by microorganisms of different phenol concentration and different inoculum volume. The step for this experimental procedure is:

- Prepare minimal salt media (MSM) in 2 liter of Erlenmeyer conical flask and Measure 100ml MSM with the help of measuring cylinder and take it in a 250 ml flat bottom conical flask and autoclaved it to 121°C for 15 minutes.
- Adjust pH of the each conical using buffers (acid & base) as per requirement for the experiment.
- Inoculate phenol for each flask by diluting from the phenol stock solution as and vary phenol concentration form 10 ppm to 50pp and check pH each of the conical.
- Inoculate 10µl of fungal spore to the each of the conical for the degradation for the study and incubate it at 130 rpm and different temperature for the study.
- Also the inoculum volume was changed for the study. Three different volumes of inoculum mainly 5µl, 10µl, and 20µlis used and inoculated with 100ppm of phenol.
- Take sample from each of the conical for regular interval (24 hrs.) of time and measure absorbance by spectroscopic method.

4.1.7.3 Effect of agitation and substrate utilization:

To study the effect of agitation, experiments were conducted under shaking and static conditions by keeping the inoculated cultures on a rotary shaker at 80rpm,120rpm, 140 rpm shaking speed and resting conditions. The initial phenol concentration of 100mg/l, pH adjusted to 5 and at a temperature of 30°C was

used for this study. The phenol degrading ability of fungi was investigated by testing the shaking speed compared to static conditions. The effect of substrate utilization as the carbon source on the phenol degrading ability of fungi is invested under condition.

4.2 Results:

4.2.1 Pre-isolated fungi:

Figure 7: Two pre isolated fungi Alternaria alternata (F-1) and Aspergillus niger (F-9)

These are the two pre isolated fungi collected from Sunderban, West Bengal which is made to grow on Czapek Dox Agar media and subcultured using streak plate method for further study. After Subculturing these fungi are made to grow on minimal salt agar plate containing variable phenol concentration ranging for 50ppm to 500ppm

4.2.2 Subcultured fungi:

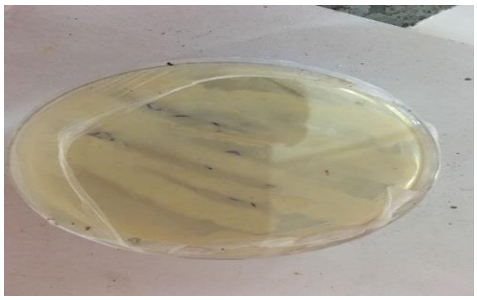


Figure 8: Subcultured Alternaria Alternata (F-1)

Figure 9: Subcultured Aspergillus niger (F-9)

Above fungi shown in figure is obtained after Sub culturing from the mother plates. These are again made to grow on the minimal salt media (MSM) with different phenol concentration ranging from 50-500 ppm to check viable capacity of fungi in the minimal salt media (MSM).These are incubated 30°C for 3 days.

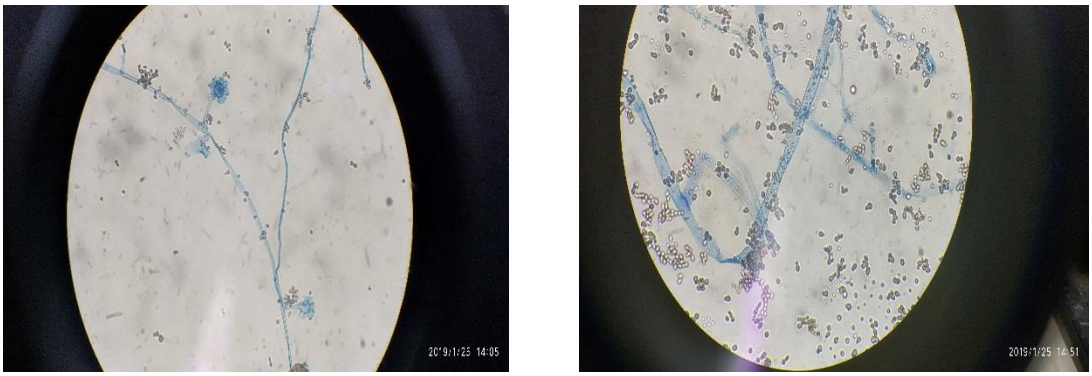


Figure 10: Fungi under microscope (60X)

A long, branched, filamentous structure of mycelium growth of fungi shows its hyphae structure under the microscope at 60x.

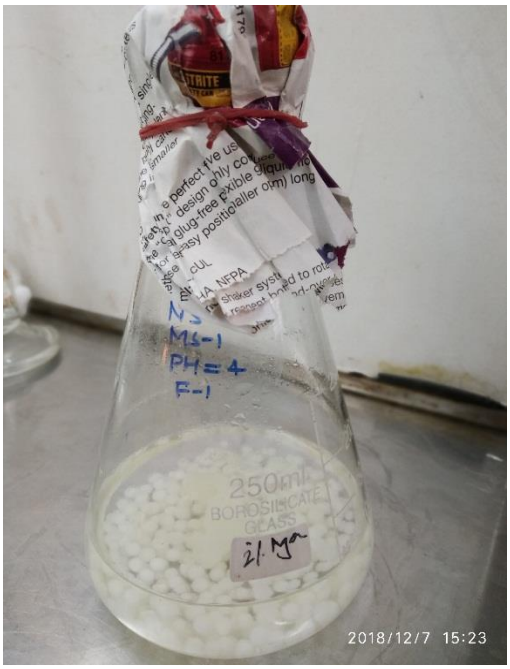


Figure 11: Alterneria alternata(biomass)

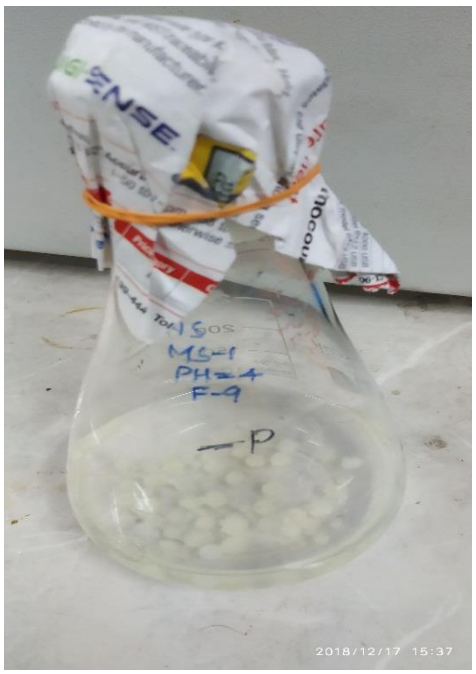


Figure 12: Aspergillus niger(biomass)

Figure shows the fugnal biomass that grown in the conical flask at pH 4, temperature 30° and agitation speed of 130 rpm incubated for 3 days. These are made to grown at phenol concentration of 100pm.

4.2.3 Calibration curve for phenol concentration vs absorbance:

The values for calibration curve of the concentration of phenol from 100-1000ppm and its respective absorbance are as follows:

Calibration curve values for concentration 5-500ppm

SL.No	Phenol concentration(ppm)	Absorbance(510nm)
1.	5	0.0166
2.	10	0.0763
3.	25	0.1983
4.	50	0.3831
5.	75	0.5610
6.	10	0.7610
7.	200	0.9134
8.	300	1.2856
9.	500	1.53145

Table 4: Calibration curve for phenol concentration 5-500ppm:

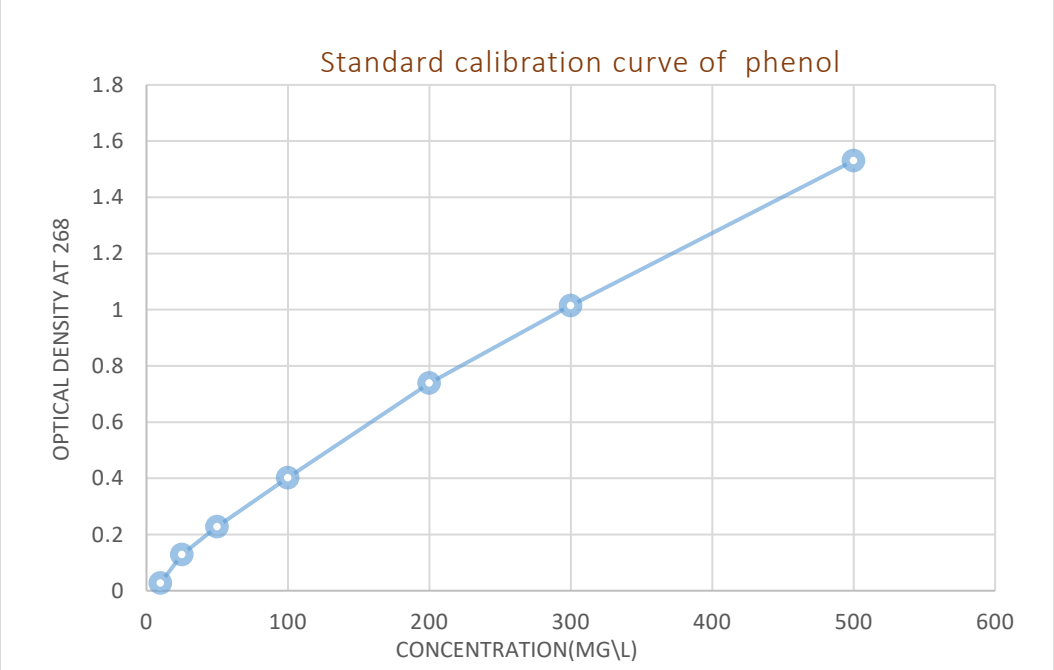


Figure 13: Calibration curve for phenol concentration

4.2.4 Spore count using hemacytometer:

Calculation of spore count using the equations:

Spores\mL= (n)*10^4

Where n= the average cell count per square pf the four corner of the square counted

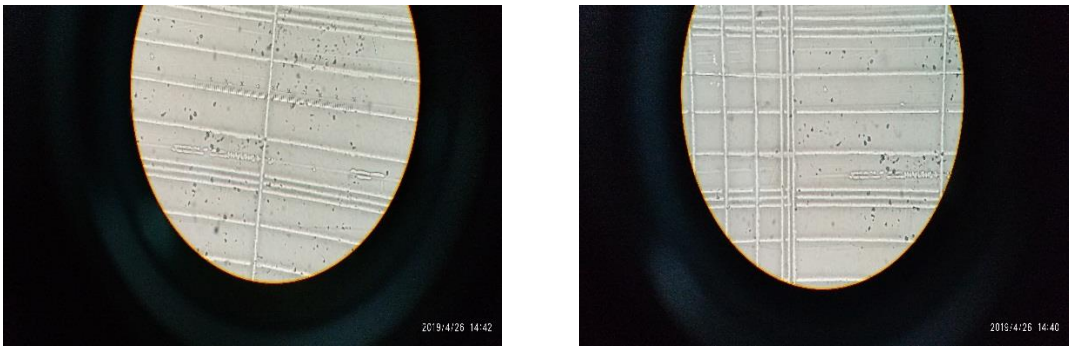


Figure 14: Microscopic fungi spore count using hemacytometer (60x)

Aspergillus niger spore count:

1st corner= 68,2nd corner= 87, 3rd corner= 58 and forth comer= 85:

$$\text{Spore\mL} = \frac{\text{1st corner+2nd corner+3nd corner+4nd corner}}{4} * 10\text{^}4$$

$$= \frac{68+87+78+85}{4} * 10\text{^}4 \text{ spores\ml}$$

$$= 7.29 * 10\text{^}6 \text{ spores\mL}$$

Alternaria alternata spore count:

1st corner= 45,2nd corner= 55, 3rd corner= 46 and forth comer= 54:

$$\text{Spore\mL} = \frac{\text{1st corner+2nd corner+3nd corner+4nd corner}}{4} * 10\text{^}4$$

$$= \frac{42+55+46+54}{4} * 10\text{^}4$$

$$= 4.93 * 10\text{^}5 \text{ spores\mL}$$

4.2.5 Phenol degradation at variable pH:

In this experimental procedure we examined the phenol degradation with respect to pH variations. Eight setups are made certainly of pH 2, 3, 4, 5.6.7.8 and 9 respectively and two strain strains of microorganisms are tested for the degradation of phenol at different pH.. The initial concentration of phenol is taken to be 100ppm

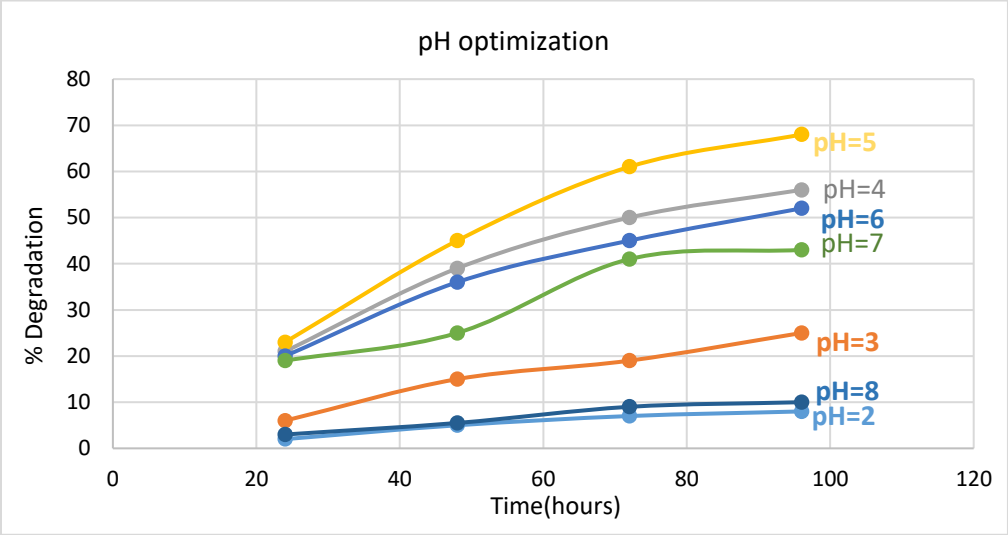


Figure 15: phenol degradation growth curve with respect to pH variation for Alternaria alternata.

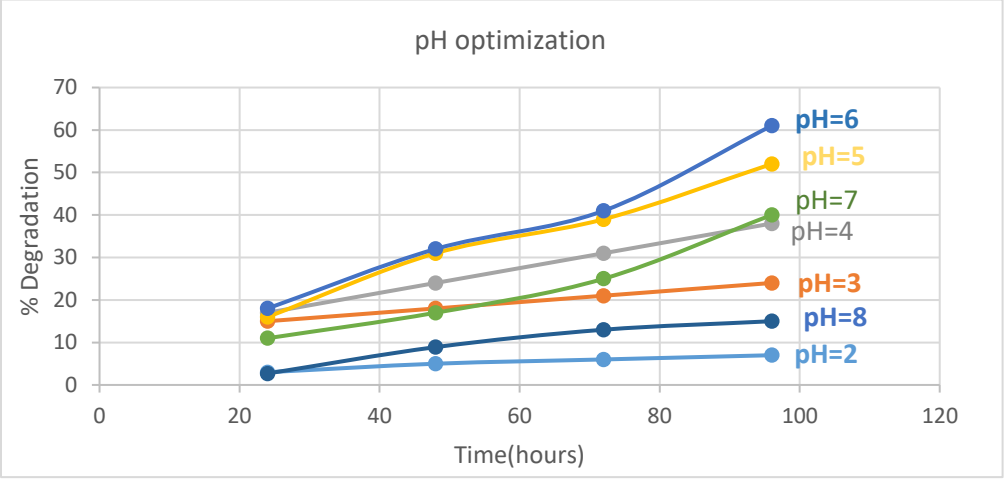


Figure 16: phenol degradation growth curve with respect to pH variation for Aspergillus niger

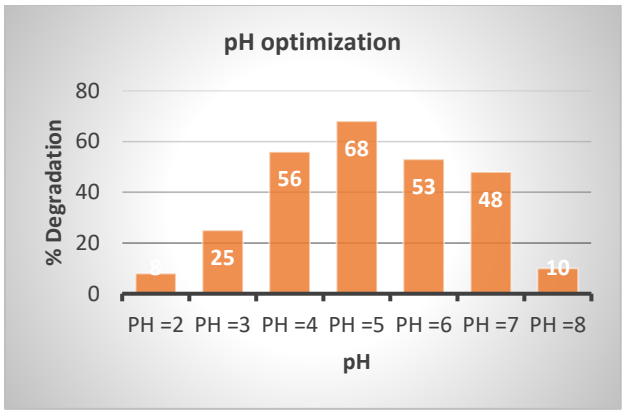


Figure 17: optimum pH for Alternaria alternata

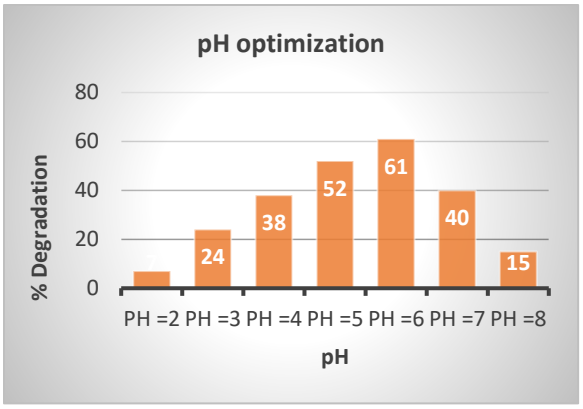


Figure 18: optimum pH for Alternaria alternata

From the above figure maximum degradation of phenol for the fungi Alternaria alternata is found to be at ph 6 and for Aspergillus niger it is at pH 6.

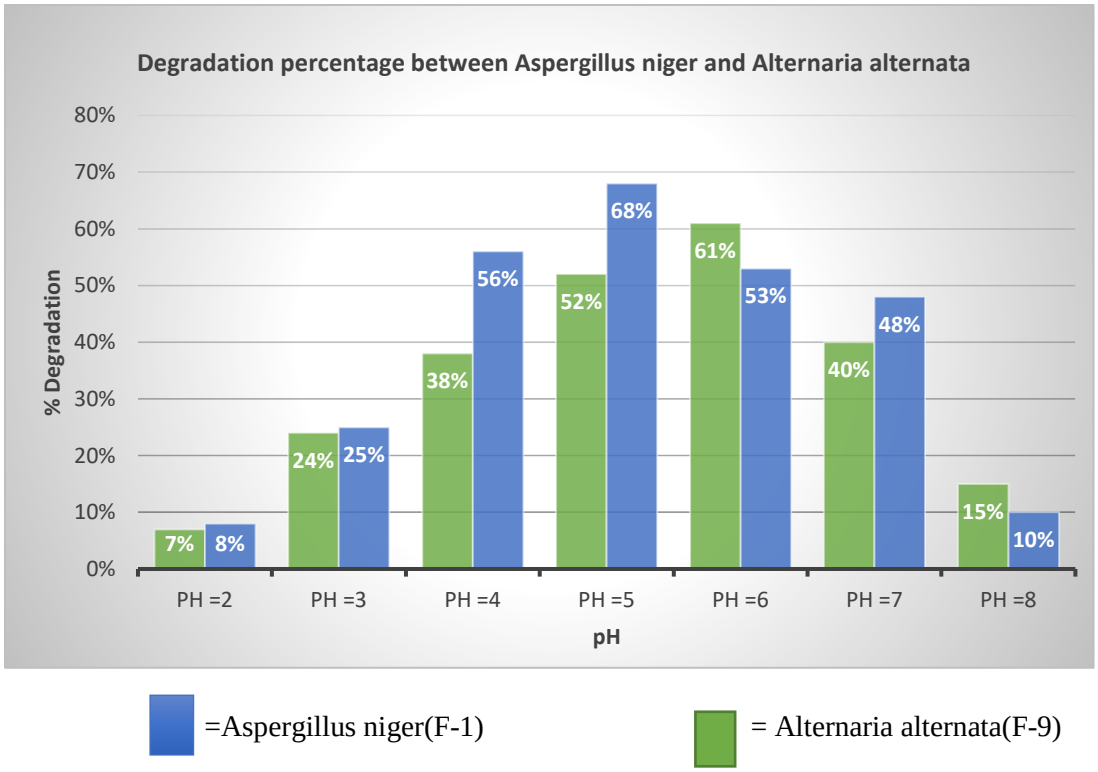


Figure 19: Degradation percentage between Aspergillus niger and Alternaria alternata

4.2.6. Phenol degradation at variable Temperature:

Temperature is very important factor for degradation in the minimal salt media (MSM) containing phenol. Here we chose three diffent temperature 25°C, 35°C and 45°C with initial phenol concentration of 100 ppm and incubated for three days with agitation of 130 rpm.

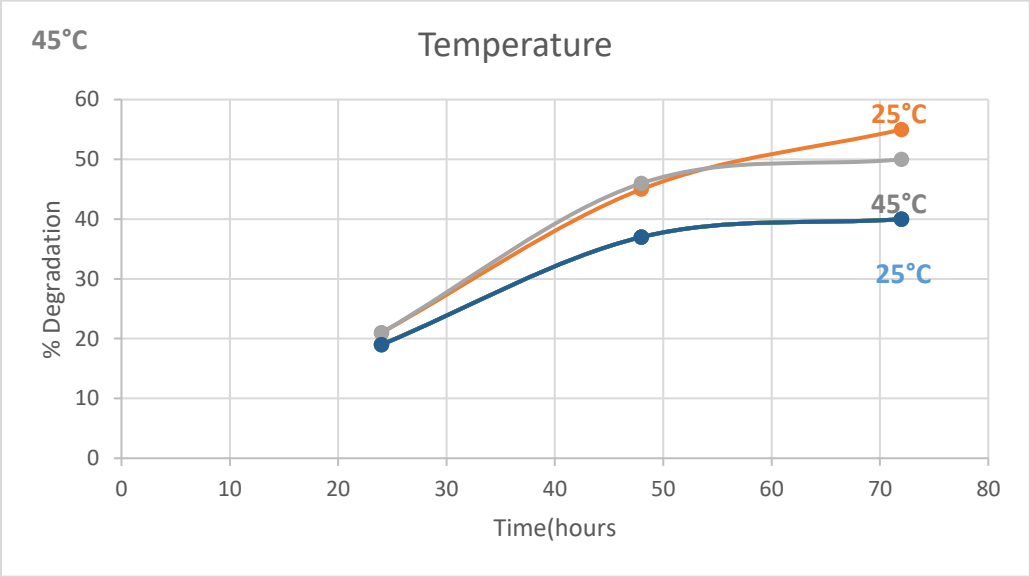


Figure 20: phenol degradation growth curve with respect to variable temperature for Alternaria alternata

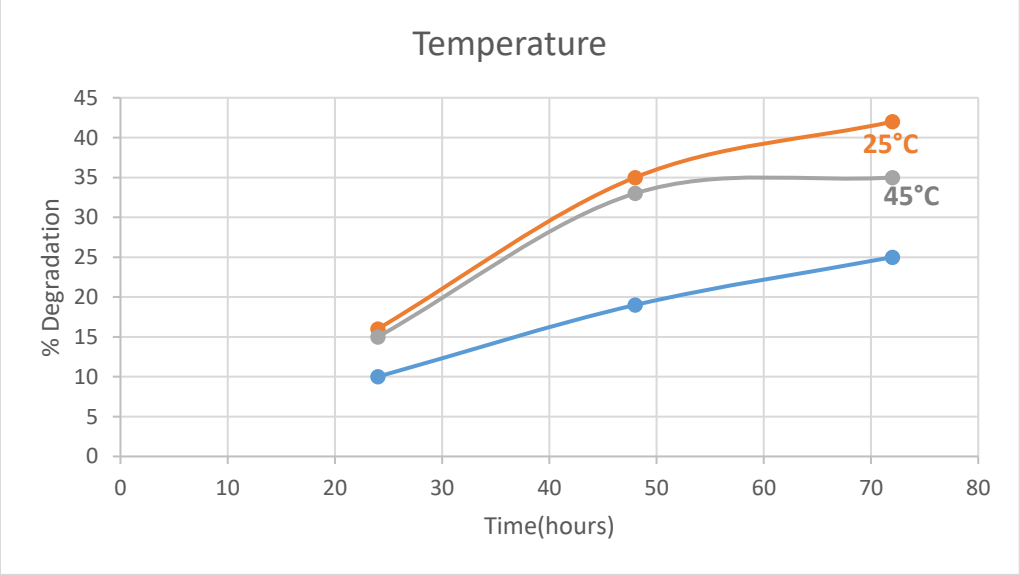


Figure 21: phenol degradation growth curve with respect to variable temperature for Aspergillus niger

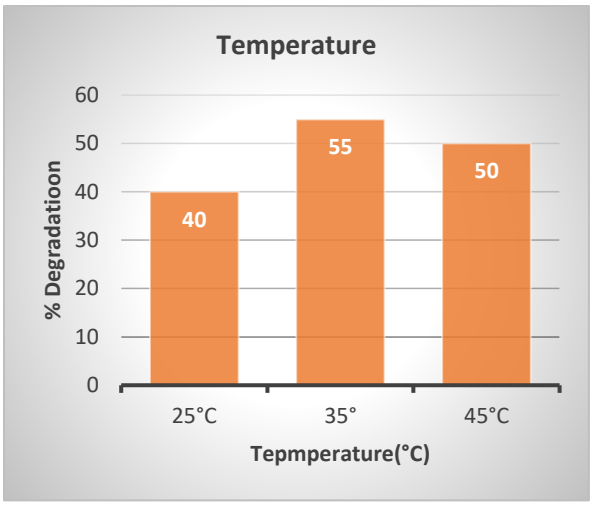


Figure 22: Optimum growth at variable temperature of Aspergillus niger(F-1)

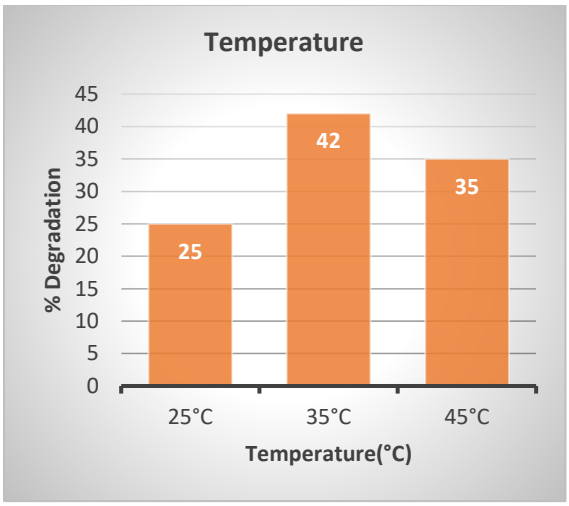


Figure 23: Optimum growth at variable temperature of Alternaria alternata(F-9)

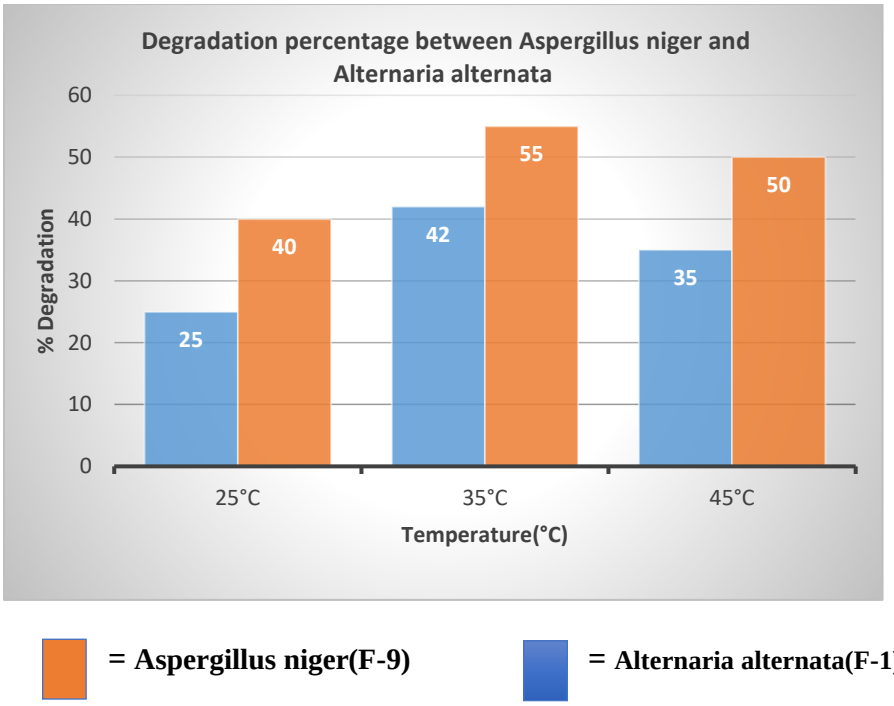


Figure 24: Degradation percentage between Aspergillus niger and Alternaria alternata

4.2.7 Phenol degradation at variable phenol concentration:

In this experiment we check the effect on growth at different phenol concentration and hence to check the ability of organism in presence of phenol

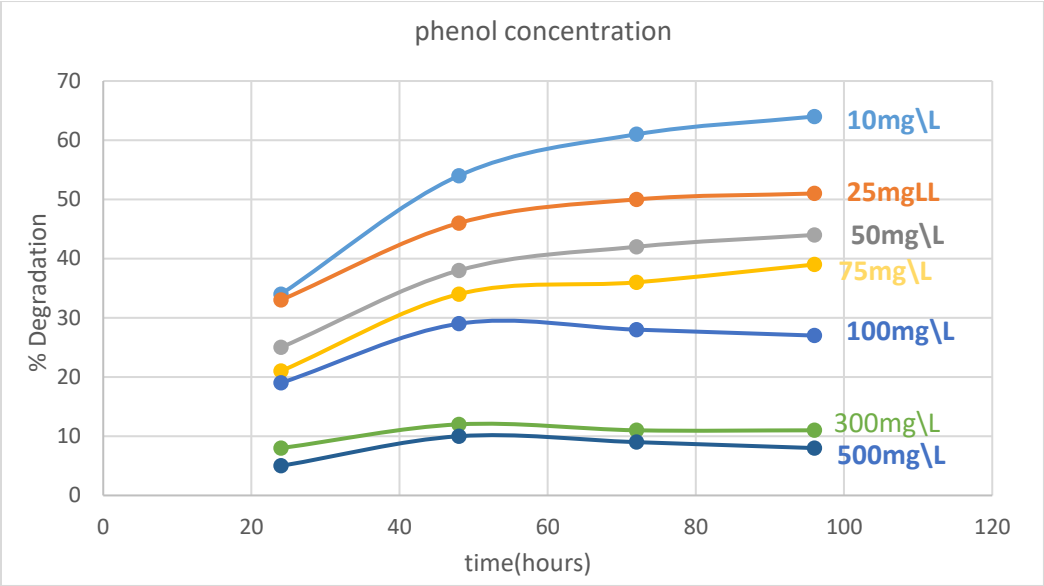


Figure 25: phenol degradation growth curve with respect to variable phenol concentration for Alternaria alternata

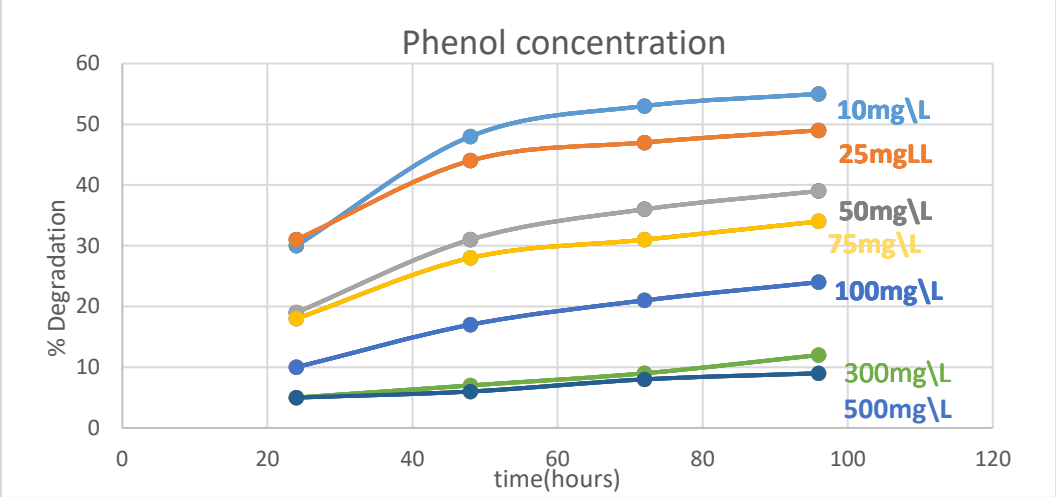


Figure 26: phenol degradation growth curve with respect to variable phenol concentration for Aspergillus niger

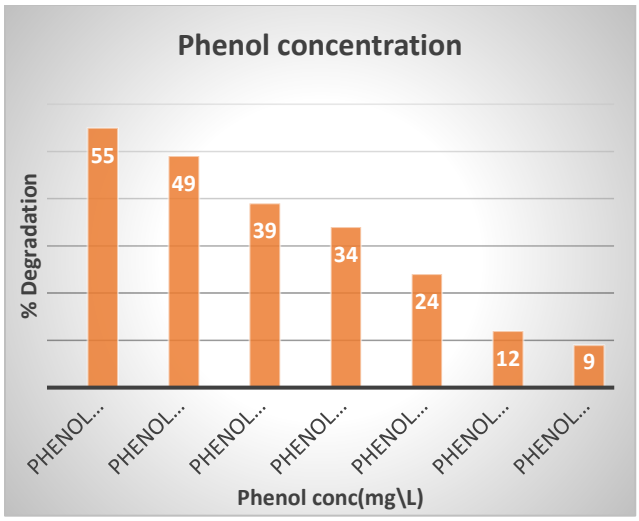


Figure 27: optimum phenol conc. for Alternaria alternata

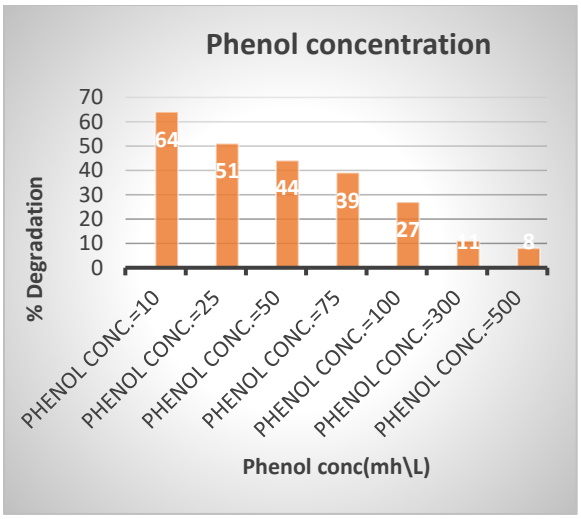


Figure 28: optimum phenol conc. For Aspergillus niger

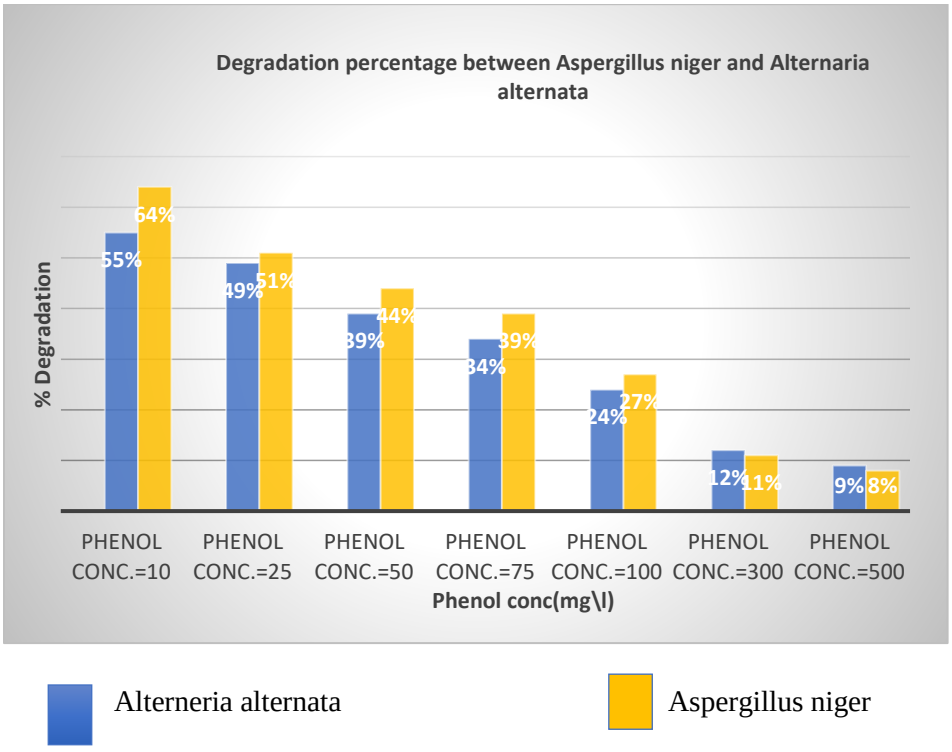


Figure 29: Degradation percentage between Aspergillus niger and Alternaria alternata

4.2.8 Phenol degradation at variable inoculum size:

Inoculum of different ages were used to inoculate the media was made study on the degradation pattern given by inoculum of different sizes with the initial phenol concentration of 100ppm.

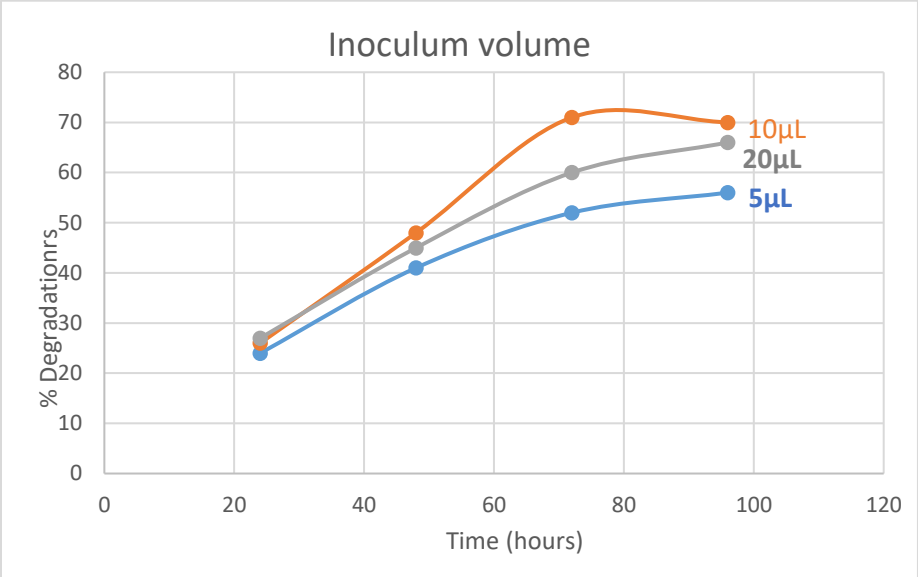


Figure 30 : Phenol degradation growth curve with respect to variable inoculum size for Alternaria alternata

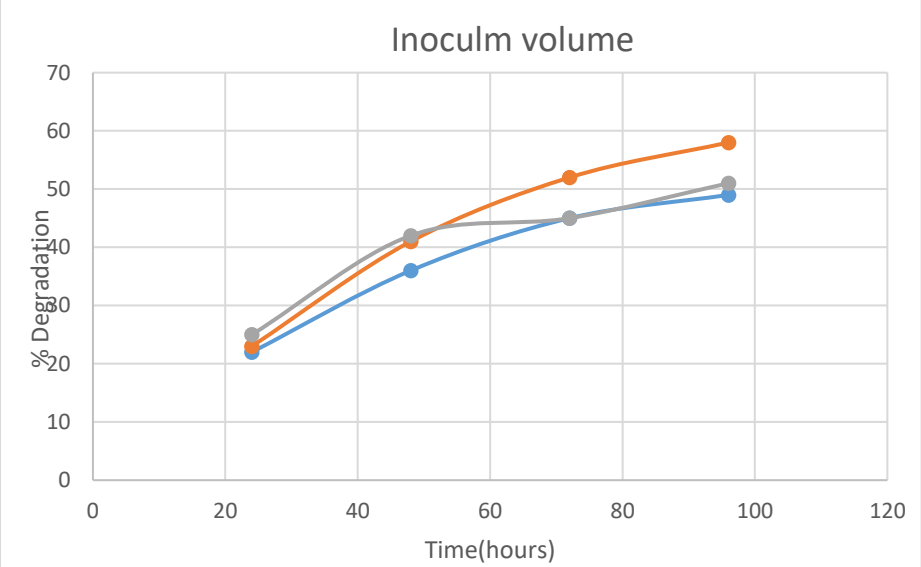


Figure 31: Phenol degradation growth curve with respect to variable inoculum size for Aspergillus niger

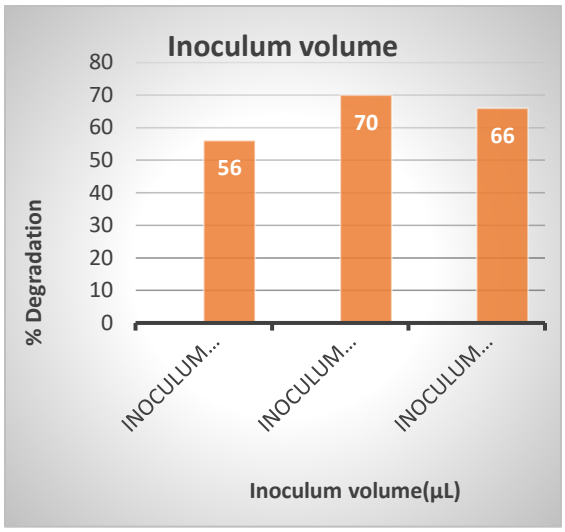


Figure 32: Optimum inoculum vol. for Alternaria alternata

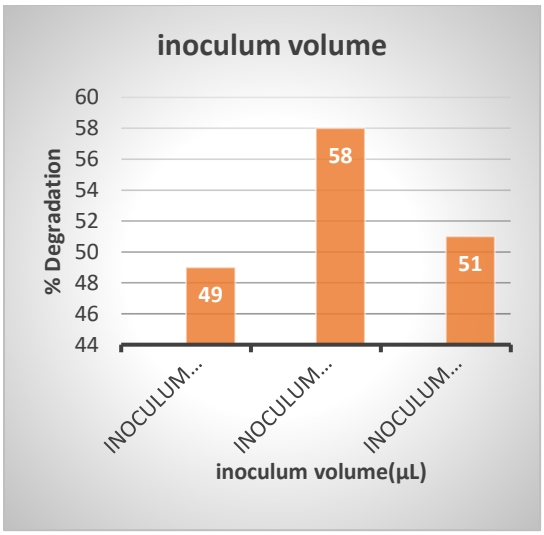


Figure 33: Optimum inoculum vol. for Aspergillus niger

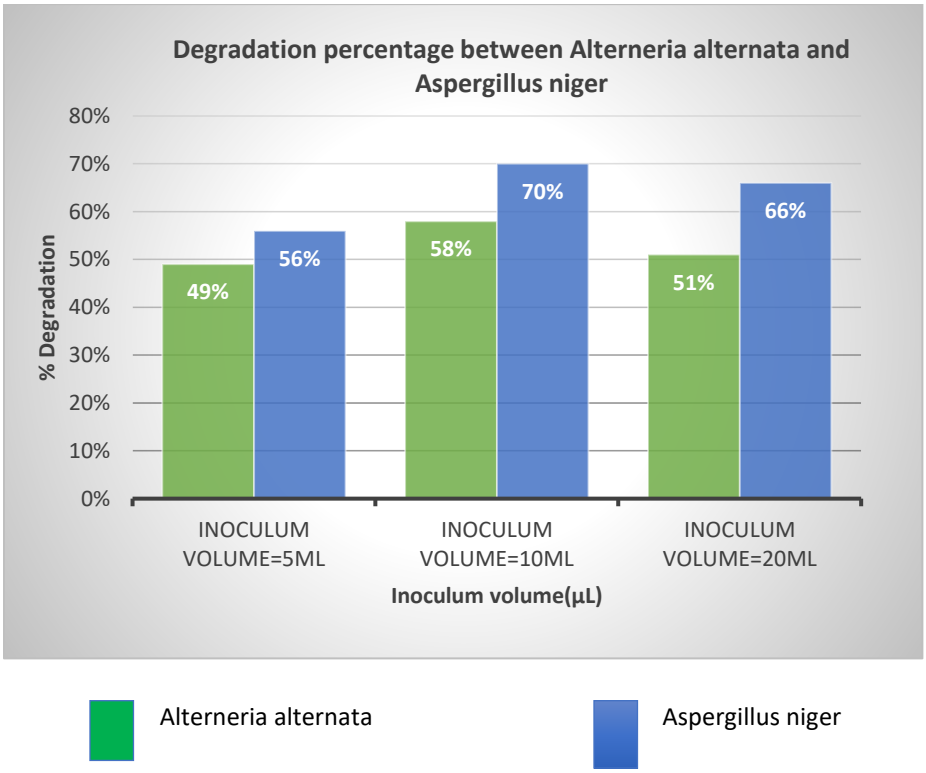


Figure 34: Degradation percentage between Alternaria alternata and Aspergillus niger

4.2.9 Phenol degradation at variable Agitation:

Agitation speed is one of the dependent variable in the optimization process. The experiment were performed in four different variable like stagnant, 80 rpm, 120 rpm .ad 140 rpm at 30°C for 4 days

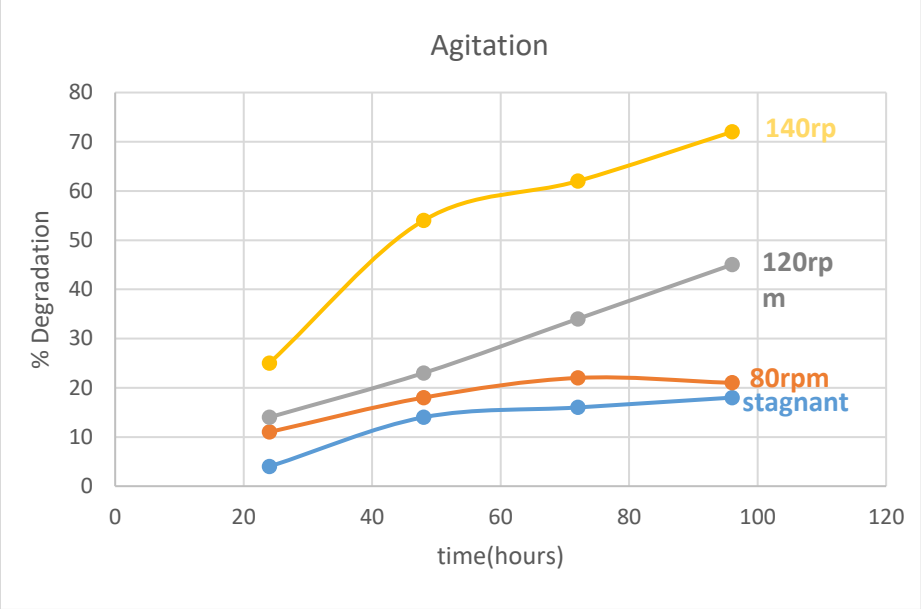


Figure 35: Phenol degradation growth curve with respect to variable agitation for Alternaria alternata

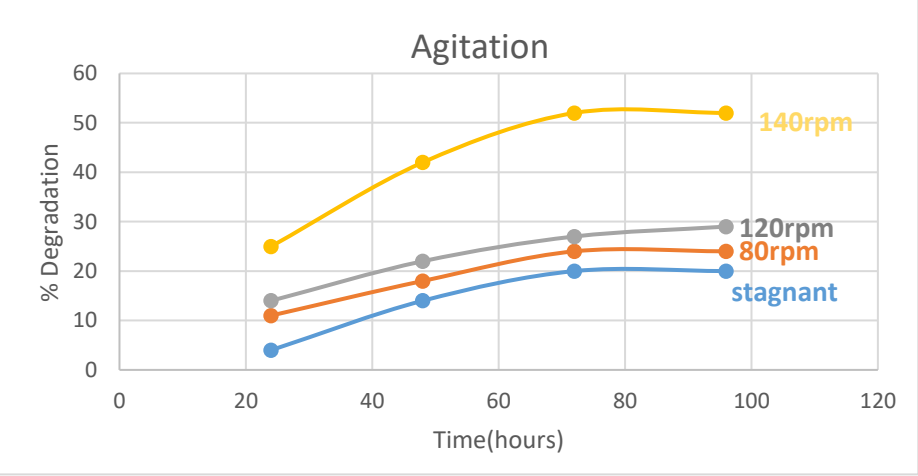


Figure 36: Phenol degradation growth curve with respect to variable agitation for Aspergillus niger

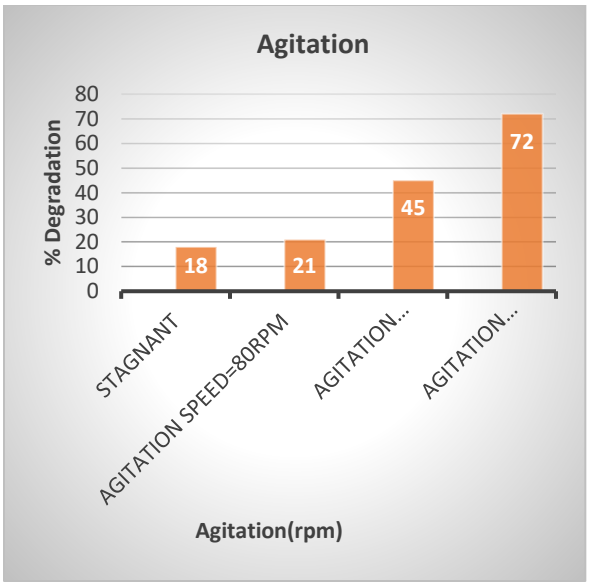


Figure 37: Optimum agitation for Alternaria alternata

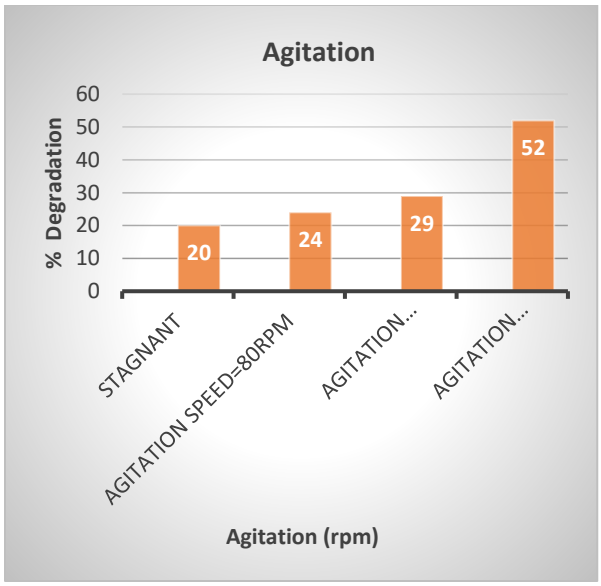


Figure 38: Optimum agitation for Alternaria alternata

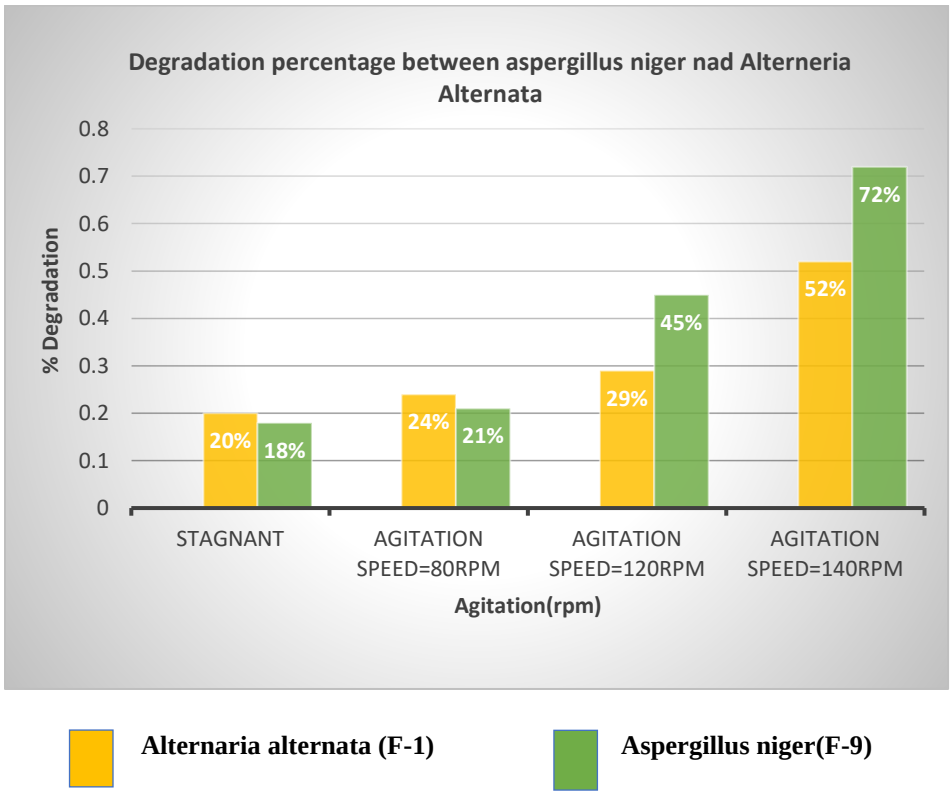


Figure 39: Degradation percentage between Aspergillus niger and Alternaria Alternata

4.2.10 Substrate effect on phenol degradation:

Substrate such as glucose sucrose accelerate the phenol degradation rate microorganisms utilize the phenol along with substrate**.**

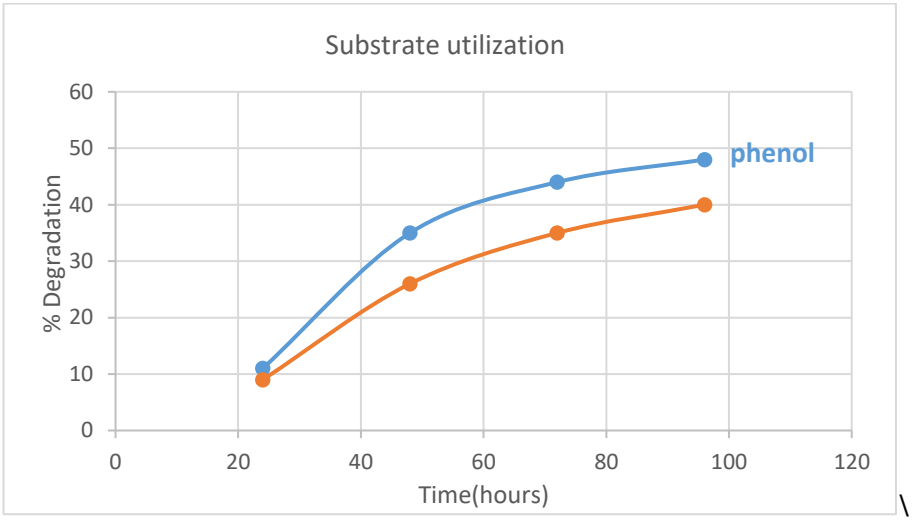


Figure 40: Phenol degradation growth curve with phenol and phenol with sucrose for Alternaria alternata

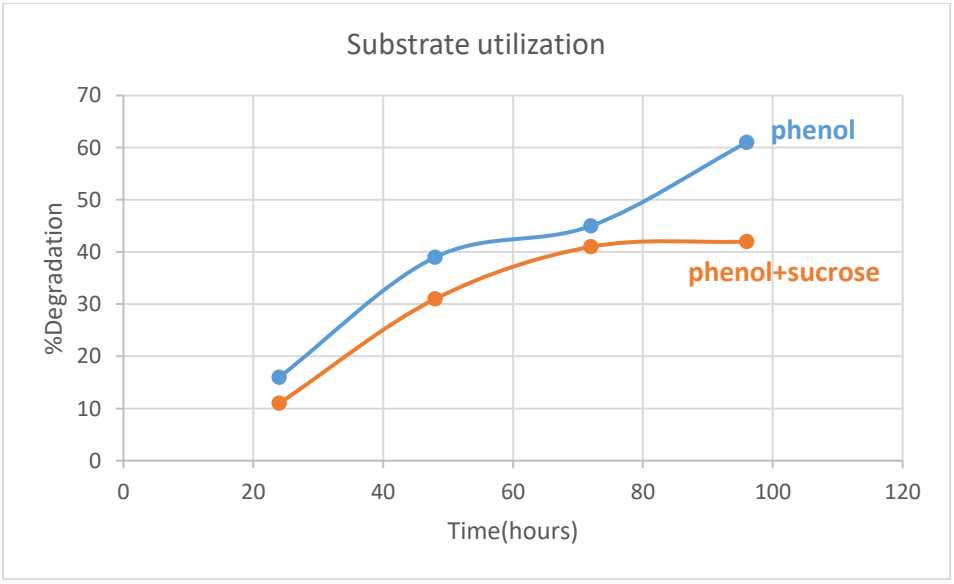


Figure 41: Phenol degradation growth curve with phenol and phenol with sucrose for Aspergillus niger

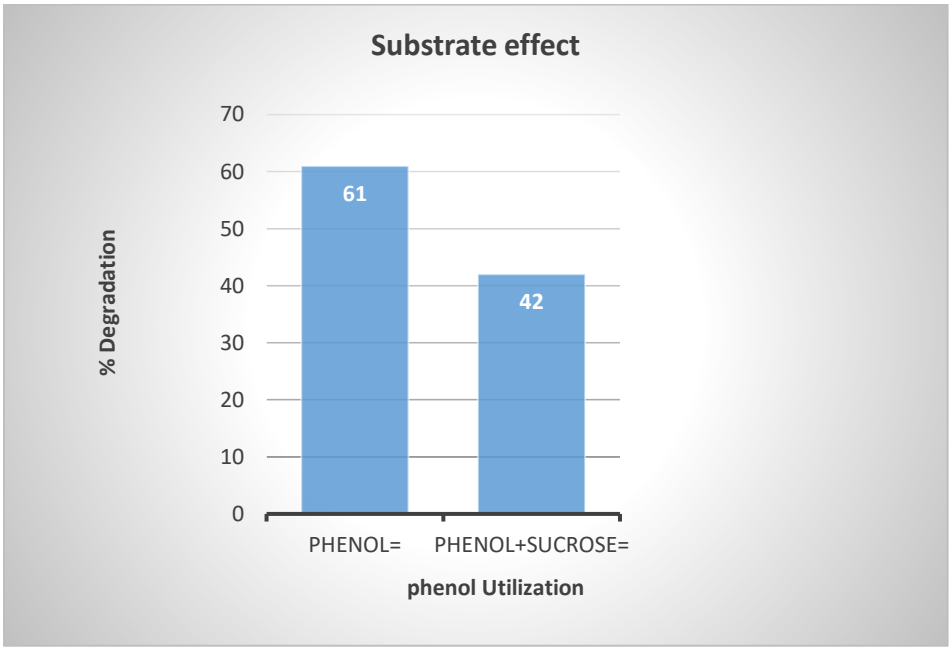


Figure 42: Phenol degradation with phenol and phenol with sucrose for Alternaria alternata

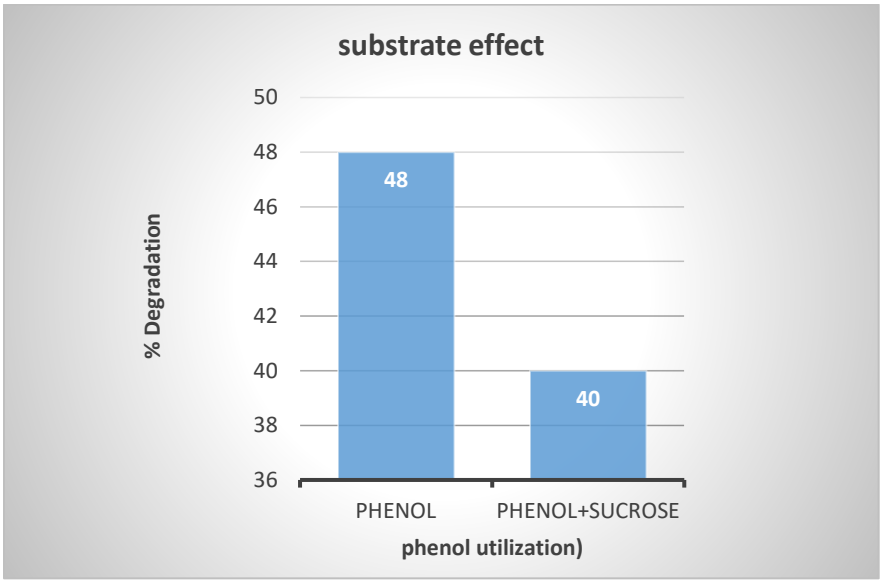


Figure 43: Phenol degradation with phenol and phenol with sucrose for Aspergillus nigers

4.2.11 Response Surface Methodology (RSM):

Response surface methodology is a statistical approaches to establish the relationships between two or more variable. RSM employed to maximize the production parameter for the special substance by optimization of operational factors such as pH, inoculum volume, temperature etc.

Optimization of process parameter using RSM for Aspergillus niger:

Std.	Run	Block 1	Factor 1 A pH	Factor 2 B: phenol conc.	Factor 3 C: inoculum vol.	Response 1 % Degradation
11	1	Block 1	6.00	32.96	7.50	66.06
13	2	Block 1	6.00	75.00	3.30	38.58
10	3	Block 1	7.68	75.00	7.50	42.41
1	4	Block 1	5.00	50.00	5.00	66.02
14	5	Block 1	6.00	75.00	11.7	62.77
12	6	Block 1	6.00	117.04	7.5	52.19
17	7	Block 1	6.00	75.00	7.50	51.02
2	8	Block 1	7.00	50.00	5.00	45.36
3	9	Block 1	5.00	100.00	5.00	43.55
20	10	Block 1	6.00	75.00	7.50	51.02
19	11	Block 1	6.00	75.00	7.50	51.02
9	12	Block 1	4.32	75.00	7.50	63.38
16	13	Block 1	6.00	75.00	7.50	51.02
7	14	Block 1	5.00	100.00	10.00	55.13
6	15	Block 1	7.00	50.00	10.00	62.72
15	16	Block 1	6.00	75.00	7.50	51.02
18	17	Block 1	6.00	75.00	7.50	51.02
8	18	Block 1	7.00	100.00	10.00	49.01
5	19	Block 1	5.00	50.00	10.00	72.21
4	20	Block 1	7.00	100.00	5.00	35.00

Table 5: Data table for optimization parameters for Aspergillus niger

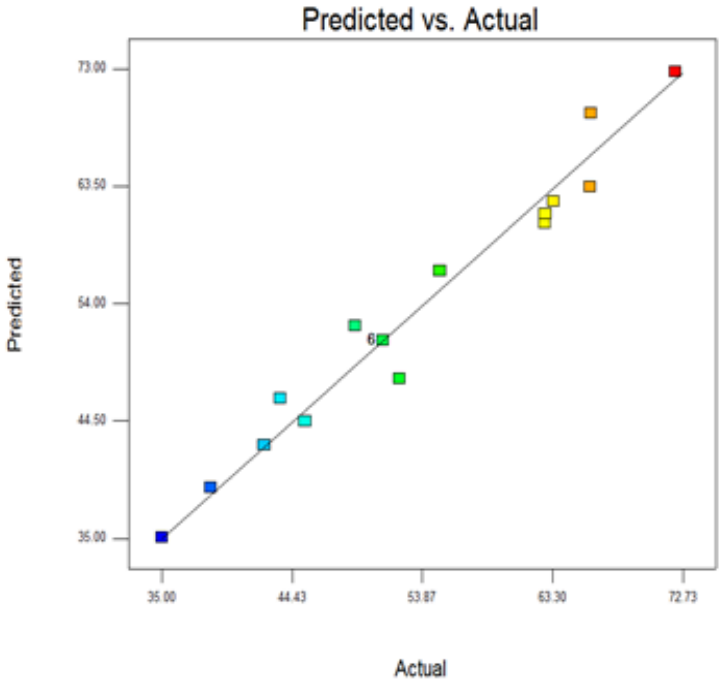


Figure 44: Predicted vs. Actual phenol biodegradation for Aspergillus niger

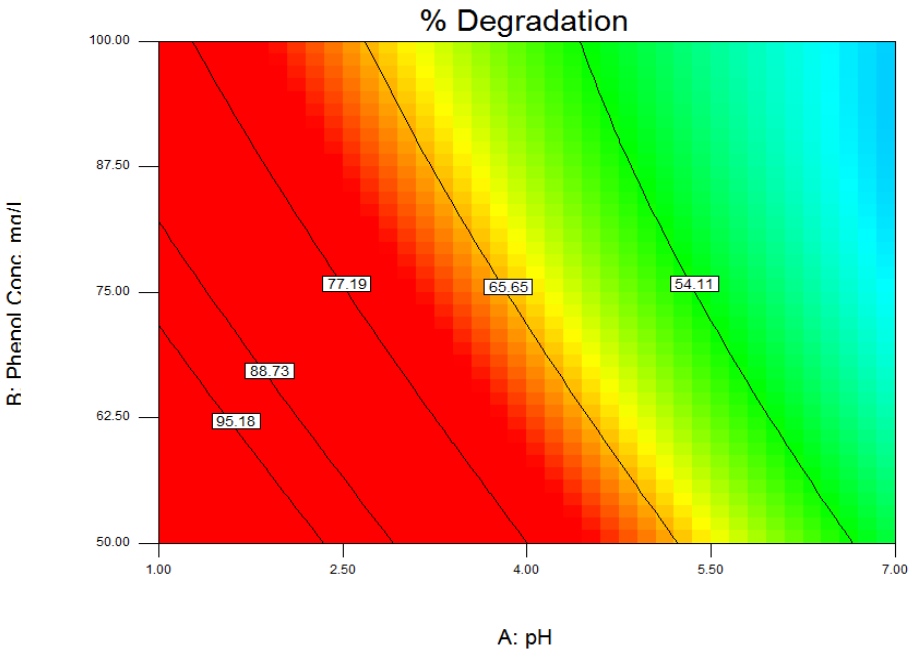


Figure 45: Phenol degradation at variable pH and phenol concentration

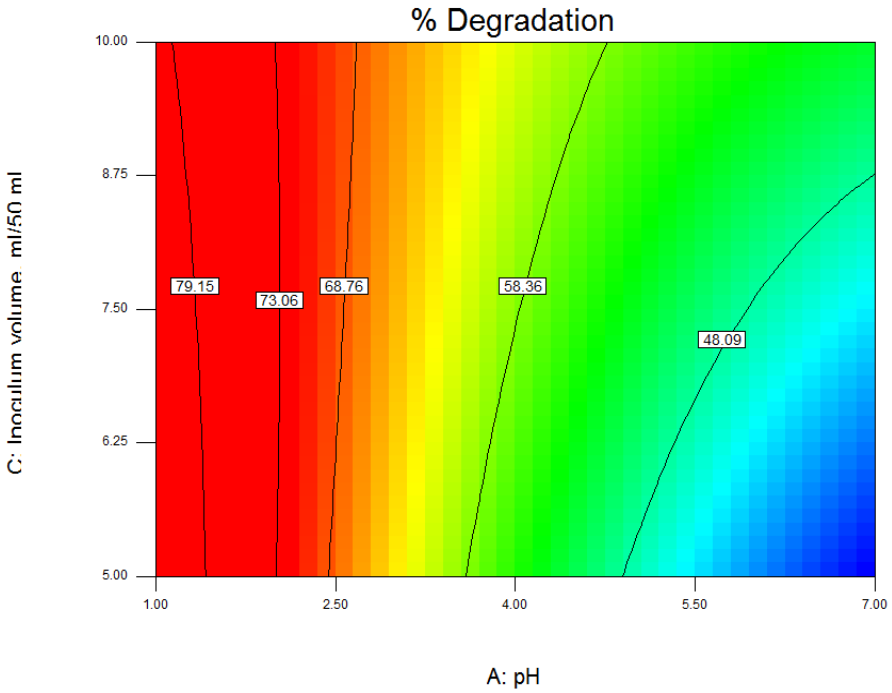


Figure 46: Phenol degradation at variable pH and inoculum volume

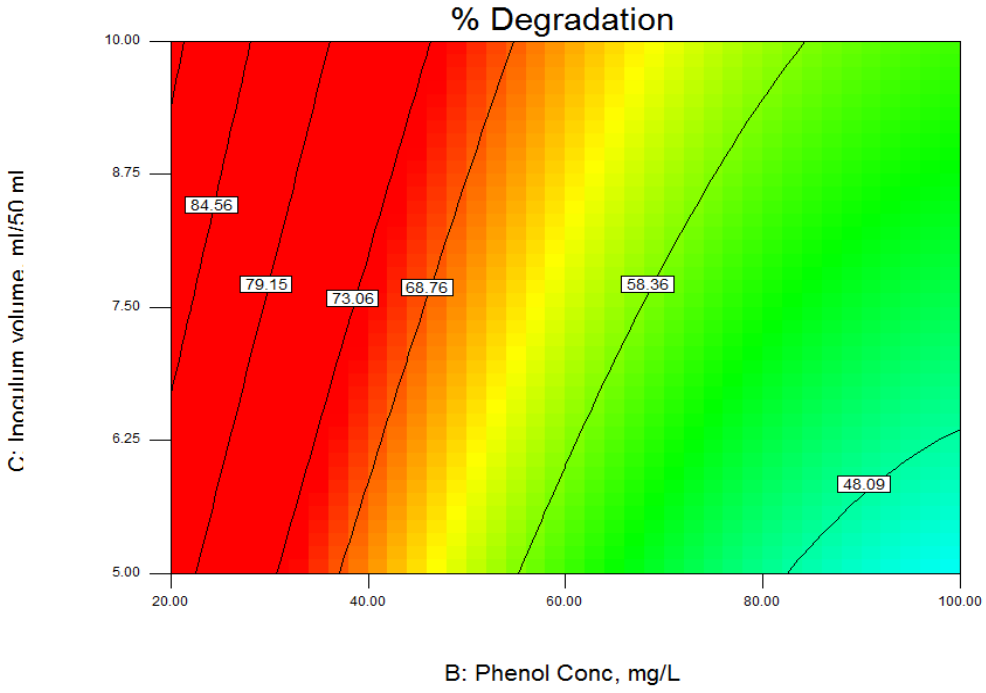


Figure 47: Phenol degradation at variable inoculum volume and phenol concentration

Optimized process parameter is as follows:

pH 3, conc: 40 ppm, inoculum volume: 8

Response 1% Degradation
ANOVA for Response Surface Quadratic Model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of square	df	Mean square	F value	P value Probe>F
Model	1779.10	9	197.683	0.31	<0.0001 significant
A-pH	469.62	1	469.62	72.02	< 0.0001
B-Phenol	553.58	1	553.58	84.89	< 0.0001
C-Inoculum volume	591.62	1	591.62	90.73	< 0.0001

Std. Dev.2.55
R-Squared 0.9646
Mean53.02
Adj R-Squared 0.9328
C.V. %4.82

Final Equation in Terms of Actual Factors:

% Degradation =+192.75622-22.71359* pH-1.39719* Phenol Conc-1.04148 *Inoculum volume+0.077415 * pH * Phenol Conc+0.68015* pH * Inoculum volume+4.07400E-003* Phenol Conc * Inoculum volume+0.49519 * pH^2+4.31653E-003 * Phenol $Conc^2$-0.047483 * Inoculum $volume^2$

Optimization of process parameter using RSM for Alternaria alternata:

Std.	Run	Block 1	Factor 1 A pH	Factor 2 B: phenol conc.	Factor 3 C: inoculum vol.	Response 1 % Degradation
11	1	Block 1	6.00	32.96	7.50	45.39
13	2	Block 1	6.00	75.00	3.30	41.46
10	3	Block 1	7.68	75.00	7.50	34.59
1	4	Block 1	5.00	50.00	5.00	51.63
14	5	Block 1	6.00	75.00	11.7	41.46
12	6	Block 1	6.00	117.04	7.5	40.50
17	7	Block 1	6.00	75.00	7.50	34.59
2	8	Block 1	7.00	50.00	5.00	49.39
3	9	Block 1	5.00	100.00	5.00	31.59
20	10	Block 1	6.00	75.00	7.50	40.50
19	11	Block 1	6.00	75.00	7.50	51.63
9	12	Block 1	4.32	75.00	7.50	29.56
16	13	Block 1	6.00	75.00	7.50	37.57
7	14	Block 1	5.00	100.00	10.00	51.65
6	15	Block 1	7.00	50.00	10.00	26.54
15	16	Block 1	6.00	75.00	7.50	23.63
18	17	Block 1	6.00	75.00	7.50	24.24
8	18	Block 1	7.00	100.00	10.00	51.63
5	19	Block 1	5.00	50.00	10.00	34.26
4	20	Block 1	7.00	100.00	5.00	54.25

Table 6 : Data table for optimization parameters for Alternaria alternata

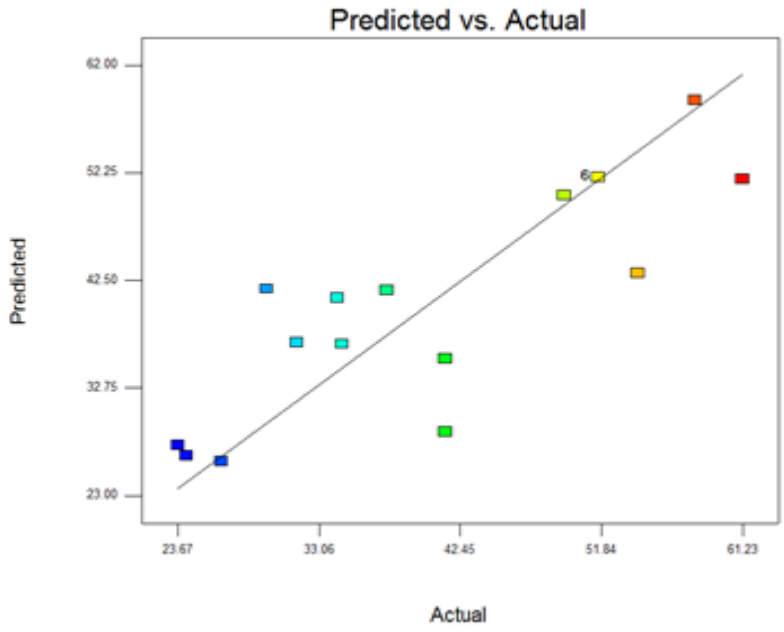


Figure 48: Predicted vs. Actual phenol biodegradation for Alternaria alternata

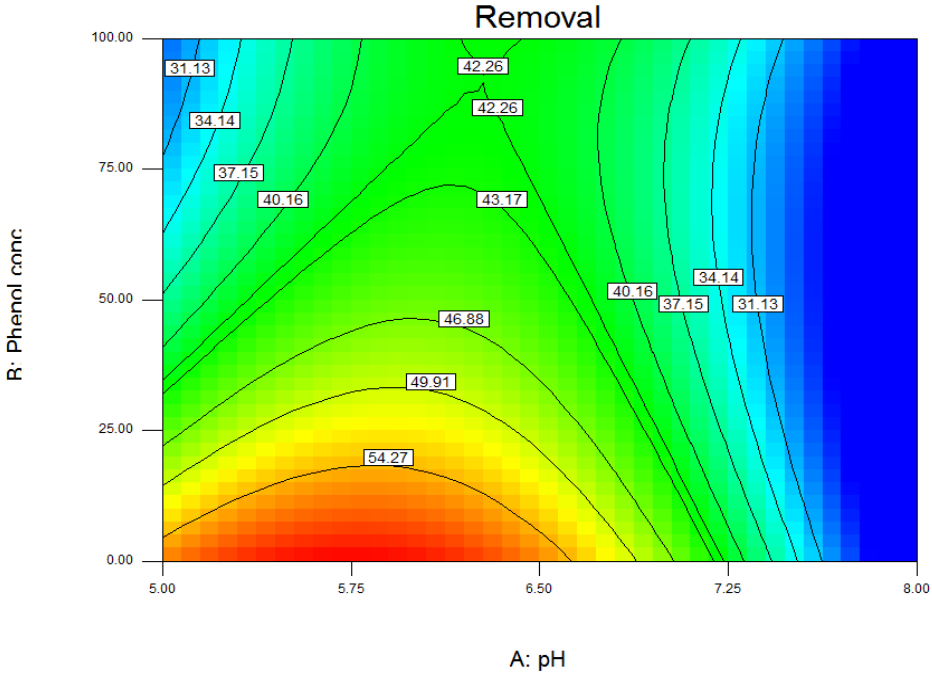


Figure 49: Phenol degradation at variable pH and phenol concentration

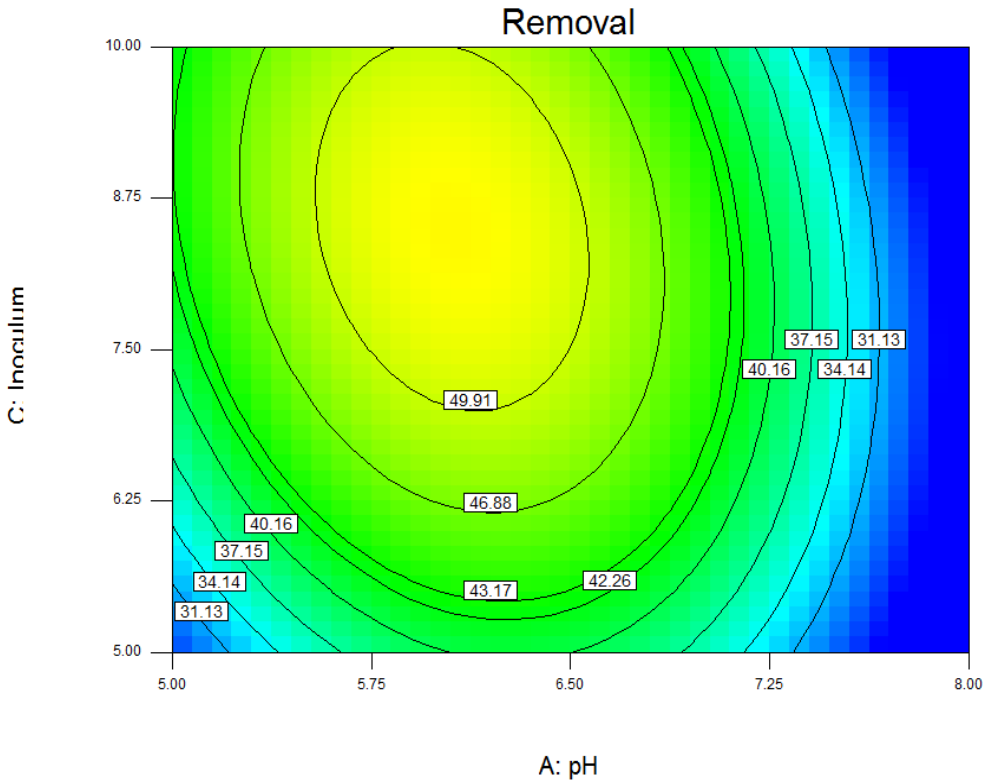


Figure 50: Phenol degradation at variable pH and inoculum volume

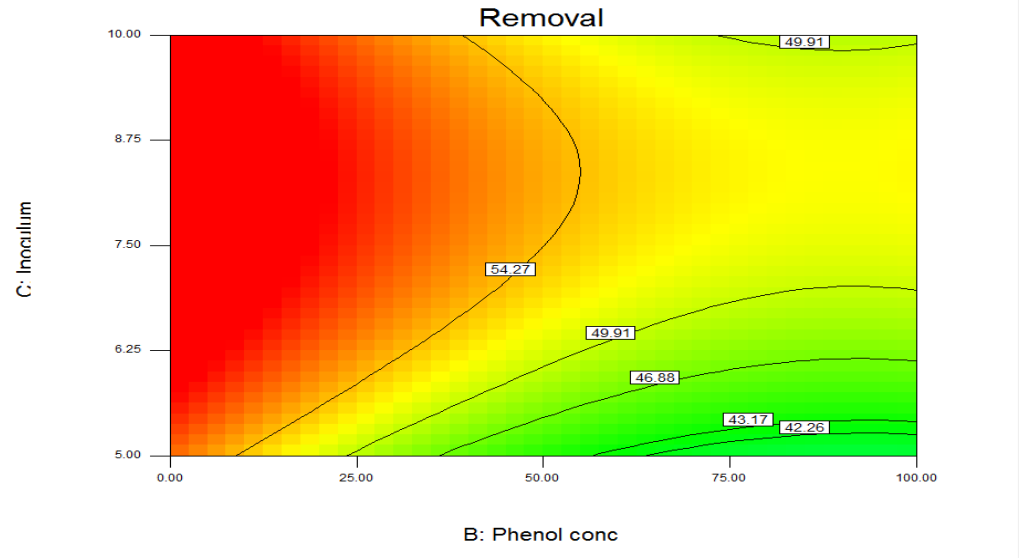


Figure 51: Phenol degradation at variable inoculum volume and phenol concentration

Optimized process parameter is as follows:

Source	Sum of square	df	Mean square	F value	P value Probe>F
Model	1986.39	9	220.71	3.27	0393 significant
A-pH	469.62	1	469.62	72.02	< 0.0001
B-Phenol	553.58	1	553.58	84.89	< 0.0001
C-Inoculum volume	591.62	1	591.62	90.73	< 0.0001

R-Squared 0.7465

Final Equation in Terms of Actual Factors:

Removal = -306.78074 +101.66381 * pH -0.96496 * Phenol conc +22.55616 *Inoculum +0.095100 * pH *Phenol conc -1.06400 * pH * Inoculum +2.84000E-003 * Phenol conc * Inoculum -8.37085 * pH^2 +1.91974E-003 * Phenol $conc^2$ -0.96146 * $Inoculum^2$

4.3 Bacterial biodegradation:

4.3.1 Required chemicals and culture media:

Phenol and chemicals, used in the study, were of analytical grade; glucose and inorganic salts, used in preparing microbial growth media, were of reagent grade. All the chemicals and reagents were procured HIMEDIA. Phenol(99% Assay) was used for the biodegradation study along with a basic mineral salt medium(MSM) broth ,Devis without Dextrose containing 1.00 g\L Ammonium sulphate $(NH_4)_2SO_4$, 7.00 g\L dipotassium phosphate (K_2HPO_4), 2.00 g\L monopotassium phosphate (KH_2PO_4)0.5 g\L Sodium citrate, 0.5 g\L Magnesium sulfate ($MgSO_4.7H_2O$)was prepared. Agar media was prepared by the addition of 28g\L Nutrient agar containing 5.0 g\L peptone, 5g\L sodium chloride (NaCl), 1.5 g\L HM peptone B#, 1.5 g\L yeast extract, and 15 g| Agar to the medium for sub culturing and nutrient broth for the degradation study. Prior to use, the media were sterilized in an autoclave at 121 °C for 15 min. The growth media were prepared by adding phenol of required concentration (10-100 mg\ L) to the MSM. Initially for isolation process, the pH of the medium was maintained at 7.00and the working volume was 100 mL in all experiments.

4.3.2. Isolation and selection of phenol degrading strain:

4.3.2.1 Sample collection for microbial isolation:

In the present study, Water samples were collected from different areas of Andaman and Digha. Water sample from Munda pahar (MP), North Bay(NB) and Randhanager(RN) of Andaman and a sample from Digha(D), West Bengal were collected. Collected sample were brought to the laboratory for the isolation and identification of bacteria using following technique.

4.3.2.2 Bacterial colony isolation using serial dilution:

The nutrient agar medium were prepared and sterilized. The medium was poured in allowed it to solidify. 1ml of the each sample in was added to 90 ml of autoclaved water in test tubes. It was shaked vigorously and 1 ml was transferred from 10^-1 dilution to next dilutions up to 10^-6.After solidifying, the nutrient agar plates with dilution 10^-4 and 10^-6 were taken.10µl of the each sample was poured in petriplates using spread plate technique. The plates ware inoculate for the bacterial growth at 37°Cfor 24 hrs. After the incubation the plates were observed.

4.3.2.3 Isolation of pure bacterial colony:

After isolating bacterial colony pure culture were prepared using streak plate method. The nutrient agar medium were prepared and sterilized. The medium was poured in allowed it to solidify. After solidification, streak one loop of isolated culture from isolated culture and streak into plates using streak

plate technique for each sample. The plates ware inoculate for the bacterial growth at 37°Cfor 24 hrs. After the incubation the plates were observed.

4.3.2.4 Subculture of the bacterial colony:

After isolating bacterial colony subculture is done using streak plate technique to obtaining discrete and more pure colony. This pure colony was used as stock plates for the further study.

4.3.3 Screening of phenol degrading strain:

For selecting the bacterial strain among four bacterial sample, we need to grow bacteria at different phenol concentration ranging from 50pp to 500ppm. This can be done using Minimal salt agar plates where phenol is used as sole source. By varying phenol concentrating, we can screened bacterial growth at higher phenol concentration hence, it can be used as degradation study.

4.3.4 Preparation of stock solution:

For preparing stock solution of phenol at any concentration we need to have the phenol of purity grade. Phenol of 100% concentration had been prepared as stock solution. Stock solution is very useful to prepare phenol solution at very low concentration. For example, if we want to prepare it from the 100% concentrated phenol in 100ml of solution, a minute amount needs very costly calibrated pipettes but most importantly it needs a high degree of accuracy in handling chemical. Hence if stock solution of say 1000 ppm is prepare and kept the low concentration solutions can be prepared easily.

There is calculation involved in this procedure,

Let ‘x’ be the concentration of the stock solution needed to prepare in ppm. Let ‘y’ be the volume of the pure culture needed to prepare 100ml stock solution of concentration ‘x’.Hence, from balancing we know that for molar balance to be equal we have,

$$M1*V1= M2*V2$$

$$x*100 = 100*y$$

$$y=10^4/x$$

4.3.5. Biochemical characterization:

4.3.5.1. Carbohydrate utilization test:

Principle: carbohydrate present in the media is broken down by bacteria either by oxidation of fermentation to reduce acid. A knowledge of sugars that can be broken down by organisms provide a valuable aid in diagnosis. Carbohydrates are added to a concentration of 05%- 1.0%, and any of the indicators like Andrede's indicator, phenol Red Bromothymol Blue or Bromocresol Purple is added to Peptone water. Change in the color of the media indicates acid production and presence of bubble in the Durham's tube indicates gas production.

Procedure

Inoculate the bacteria to be tested in the Peptone water tubes containing a carbohydrates, Durham's tubes and pH indicator

Incubate at 37°C for 24 hours

Change in color of the medium w or w/o gas production= positive reaction (indicates sugar utilization)

4.3.5.2. Phenylalanine Deaminase Test:

A small number of bacteria including all the proteae can determinate the aromatic amino acid phenylalanine to produce phenyl pyruvic acid. This end products detected by adding Ferric chloride solution which chelates with the phenyl pyruvic acid to produce a green color. The green fades rapidly so that test must be examined immediately.

Procedure:

Incubate the bacterium to be tested on phenylalanine Agar slants

Incubate at 37°C for 24 hours

After incubation add 1% Ferric chloride reagent to the slants showing growth. The slants which turn green in colour indicated that the organism is phenylalanine deasminase positive

4.3.5.3 Urease test:

Some bacteria produce the enzyme Urease. Urease splits urea into ammonia nad carbon dioxide, making the two products available for bacteria use. The test for urease production relies on the fact the ammonia produced upon hydrolysis is alkaline.

The test organism in inoculated into a urea agar that contains phenol Red, a pH indicator, and has a pH of 6.8. at this pH .Phenol Red is salmon color. However, when the pH rises above8.1, Phenol red turns a cerise (hot pink) color. Organisms that produces urease will turn cerise due to the ammonia produced upon hydrolysis of urea (positive result). Organisms that are unable to synthesize urease will not produce ammonia and thus will not experience the subsequent rise in pH .Thus a negative test is indicated by the continuance of a salmon color in the urea broth.

Procedure

Inoculate the bacteria to be tested in Urea Agar slant

Incubate at 37°C for 24 hours

After incubation the slants which turn cerise in colour indicates urease positive

4.3.5.4 Malonate Utilization Test:

This test detects the ability of an organism to utilize sodium malonate

Procedure:

Inoculate the bacterium to be tested on malonate

Incubate at 37°C or 24 hours

Change in colour of the medium from green to blue indicate utilization of sodium malonate

4.3.5.5 Gelatin Liquefaction Test:

Some bacteria produce gelatinase enzyme which degrade or liquefies gelatin

Procedure:

Inoculate the bacterium to be tested on nutrient gelatin medium

Incubate at 37°C for 24-48 hours.

Liquefaction is tested by holding nutrient gelatin culture tubes at 4° for 30 minutes before reading the results (Gelatin melts at 24°C and is therefore liquid at 37°C)

4.3.5.6 Starch Hydrolysis Test:

Procedure:

Inoculate the bacterium to be tested on starch agar

Incubate at 37°C for 24-48 hours

Flood the surface of 48 hours old culture on the starch agar with gram's iodine

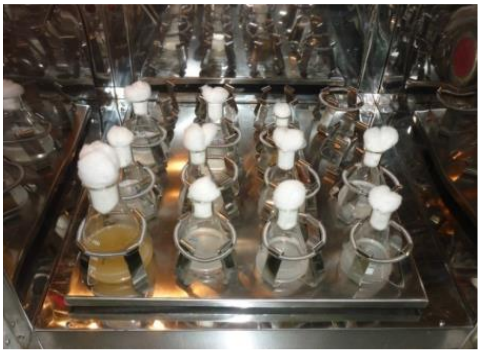
Colourless zone surrounding the colonies indicates starch hydrolysis. A blue or purple zone indicate starch is not hydrolysed.

4.3.6 Phenol biodegradation studies:

Phenol biodegradation studies:

For degradation of phenol we need to prepare the minimal salt media. The initial concentration of phenol was measured by the spectrophotometric method and absorbance was checked. Minimal salt media is prepared instead of the nutrient broth so that all the nutrients that the organisms are getting from the nutrient broth may be stopped like carbon source or nitrogen source and we can add phenol externally as

carbon source .According to the phenol concentration of solution of 100 ml needed to prepare the amount of stock solution is taken and the rest distilled water is added to make over up to 100 ml. The minimal salt media provides the conditions of minimal salt required of the growth of the microorganisms. It provides the necessary nitrogen source and the metals in trace amount like calcium and iron. Bacteria is made to grow on nutrient media in 250 ml of ml Erlenmeyer flask for the inoculum for each samples and incubate it at 35°C and 130rm for 24 hours.



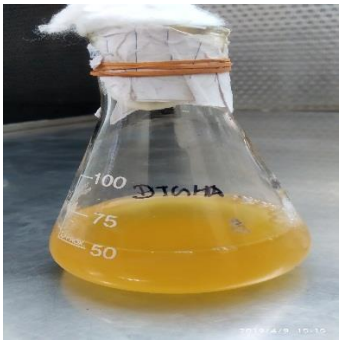


Figure 52 : Microbial growth and degradation figure 53: isolated microbes from Randhanager and digha grown in incubator.

All biodegradation experiments were performed in 100 ml Erlenmeyer flask containing 50 ml of MSM containing phenol at concentration ranging from 10 mg/L to 100 mg/L. Upon incubation of the flasks at 30°C under agitation condition (130 rpm), samples were withdrawn at regular time interval and centrifuged using laboratory centrifuge. Supernatant were analyzed under spectrophotometer (ParkinElmer) at 268 nm for phenol degradation and cell pallets were observed for microbial growth at 600nm.

4.3.7 Microbial growth kinetics for biodegradation of phenol:

For any bacteria to grow significantly, the amount of substrate must be sufficiently high as compared to the number of cells to permit sufficient growth of the microbe. If the cell density is high corresponding to the substrate concentration, little or no increase in the cells is possible. Hence it is perceivable that the extent of growth depends on the initial substrate concentration. In order to describe the kinetics of substrate degradation 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 growth of microbial culture, specific growth rates of the culture at different substrate concentrations is calculated as per the following relationship:

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

Where, m is the specific growth rate (per hour), *X* is the biomass concentration (mg/L). Usually, the microbial growth can be represented by a simple Monod equation:

$$\mu = \mu mS/(ks + S)$$

where, *S* is the limiting substrate concentration (mg/L), μmax is the maximum specific growth rate (per hour), *K*s is the half saturation constant (mg/L). On rearranging equation we get,

$$\frac{1}{\mu} = \frac{Ks}{\mu m}\left(\frac{1}{S}\right) + \frac{1}{\mu m}$$

The plots were obtained using Origin graphs and the kinetic parameters μ m , Ks were obtained using the equation For each microorganism, different μ m, Ks value is obtained and they were curved along with respective substrate concentration.

4.3.8 Batch scale study for optimization of process parameter:

4.3.8.1 Degradation of phenol at different temperature and different pH:

Degradation of phenol was studied at different temperatures below 50°C because generally existing microorganisms in nature lose their growth potential at such a high temperature. Three different temperature 25°C, 35°C and 45°C was studied to detect optimal growth of the microorganism. Also for pH the different pH ranging from pH 4 to pH 8 were used to detect the growth of microorganisms and the best one was detected with the maximum growth. The steps for the procedure is as follow:

- Prepare minimal salt media (MSM) in 2 liter of Erlenmeyer conical flask and Measure 50ml MSM in a measuring cylinder and take it in a 100 ml flat bottom conical flasks and autoclaved it to 121°C for 15 minutes.
- Adjust pH of the each conical using buffers (acid & base) as per requirement for the experiment.
- Inoculate phenol for each flask by diluting from the phenol stock solution as per the requirement and check pH each of the conical.
- Inoculate 1ml of bacterial culture to the each of the conical for the degradation for the study and incubate it at 130 rpm and different temperature for the study.

- Take sample from each of the conical for regular interval (24 hrs.) of time and centrifuge using laboratory centrifuge. Supernatant is measure under spectroscopic method for phenol degradation and cell pallets were used for the bacterial growth for each of temperature and pH study.

4.3.8.2 Degradation of phenol at different phenol concentration and inoculum volume:

Till now, the inoculation was done with the constant phenol concentration with inoculum of bacterial culture at constant volume. For examining the degradation of phenol various external factors such as pH, temperature, inoculum volume, agitation speed, phenol concentration were studied.

The basic outline of the experimental procedure is given below:

- Prepare minimal salt media (MSM) in 1 liter of Erlenmeyer conical flask and Measure 50ml MSM in a measuring cylinder and take it in a 100 ml flat bottom conical flask and autoclaved it to 121°C for 15 minutes.
- Adjust pH of the each conical using buffers (acid & base) as per requirement for the experiment.
- Inoculate phenol for each flask by diluting from the phenol stock solution as and vary phenol concentration form 10 ppm to 100ppm and check pH each of the conical.
- Inoculate 1ml of bacterial culture to the each of the conical for the degradation for the study and incubate it at 130 rpm and different temperature for the study.
- Also the inoculum volume was changed for the study. Three different volumes of inoculum mainly 0.5ml, 1.0ml, and 2.0ml is used and inoculated with 50ppm of phenol.
- Take sample from each of the conical for regular interval (24 hrs.) of time and centrifuge. Measure absorbance by spectroscopic method for degradation and growth.

4.4 Results:

4.4.1 Serial dilution for bacterial isolation:

Water samples from different areas of Andaman and Digha is collected and bring to the laboratory for isolation of single colony. Dilation up to 10^-6 to 10^-9 is made for the isolation of pure culture.

Figure 54: Isolation of pure colony using serial dilution method

4.4.2 Isolated bacterial pure culture growth:

After isolation of pure culture, these microbes are grown in the nutrient brown for the further study.

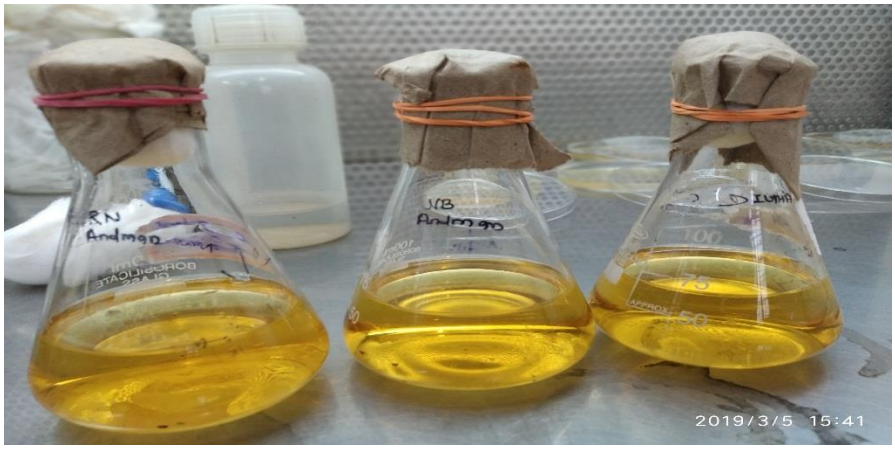


Figure 56: Isolated bacteria from different sources and their growth in incubator

4.4.3 Screening:

After isolation of the pure culture these microorganism are screened under variable phenol concentration form 50pp to 500 ppm.

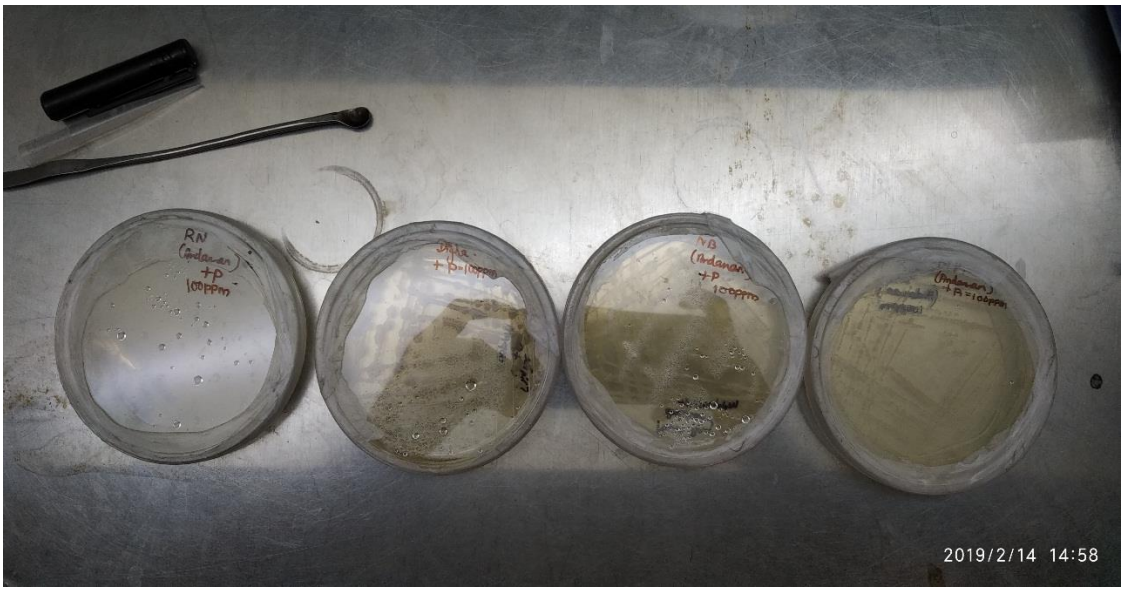


Figure 56: Isolated bacteria grown with variable phenol concentration

Collected Water samples from Andaman of different areas (Randhanager, Munda pahar, North Bay) and digha are made to grow on phenol concentration from 50ppm to 500ppm. It has been observed that water sample from digha and Randhanager can tolerate phenol concentration up to 500 ppm. So we chose isolated pure colony from Randhanager and Digha for the study.

4.4.4 Growth study:

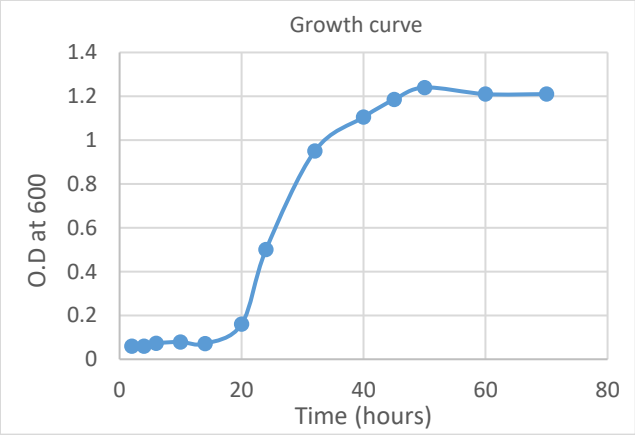


Figure 57: RN bacteria growth curve

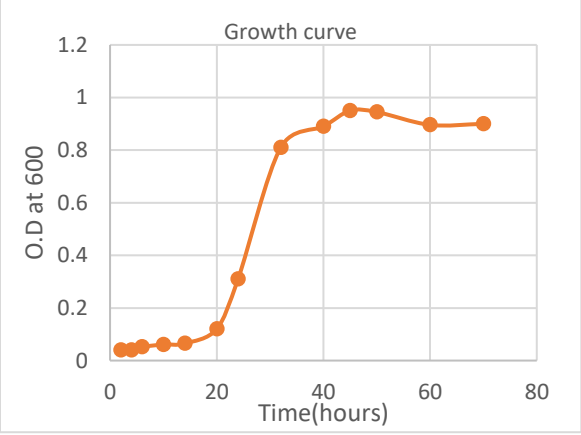
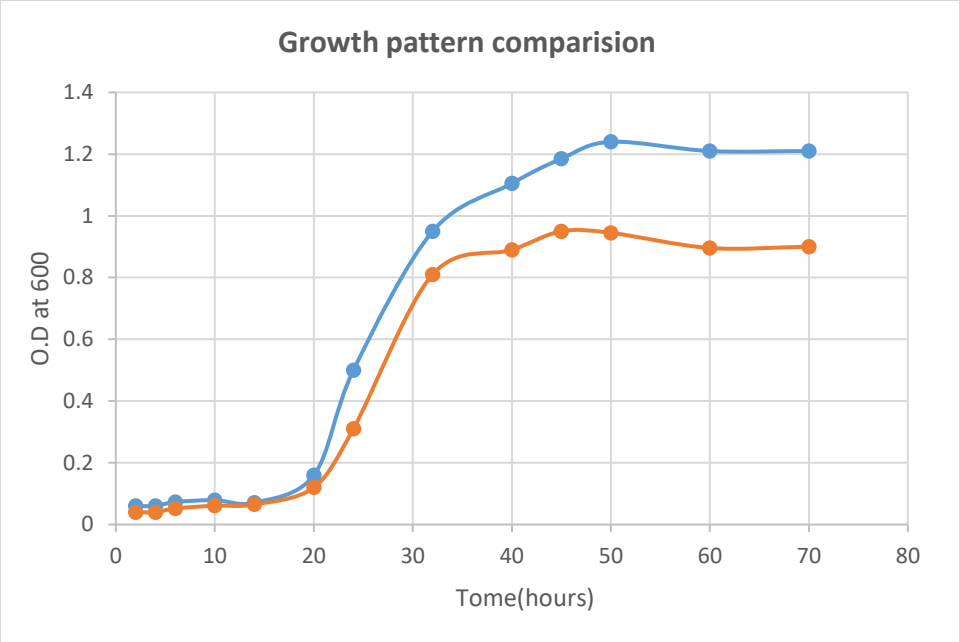


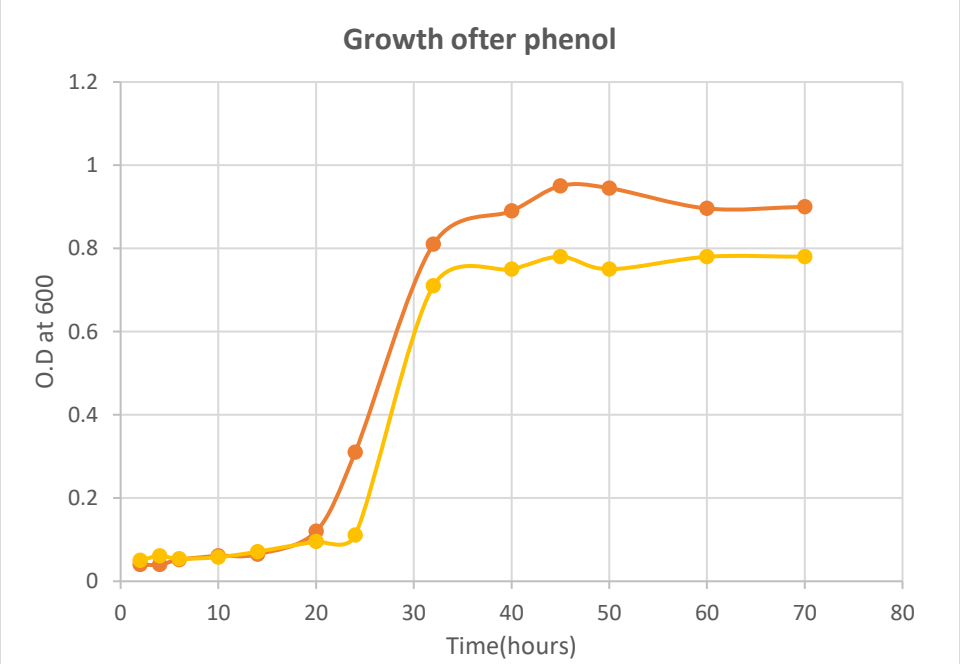
Figure 58: D bacteria growth curve

Above figure shows the bacterial growth pattern from RN and D in minimal salt media (MSM).



RN

Figure 59: Growth pattern comparison after introducing the phenol into the media.



D

Figure 60: Growth pattern comparison after introducing the phenol into the media.

4.4.5 Biochemical characteristic:

4.4.5.1 Carbohydrate utilization test:

Carbohydrate present in the media is broken down by bacteria either by oxidation of fermentation to reduce acid. A knowledge of sugars that can be broken down by organisms provide a valuable aid in diagnosis. Carbohydrates are added to a concentration of 05%- 1.0%, and any of the indicators like Andrede's indicator, phenol Red Bromothymol Blue or Bromocresol Purple is added to Peptone water. Change in the color of the media indicates acid production and presence of bubble in the Durham's tube indicates gas production.

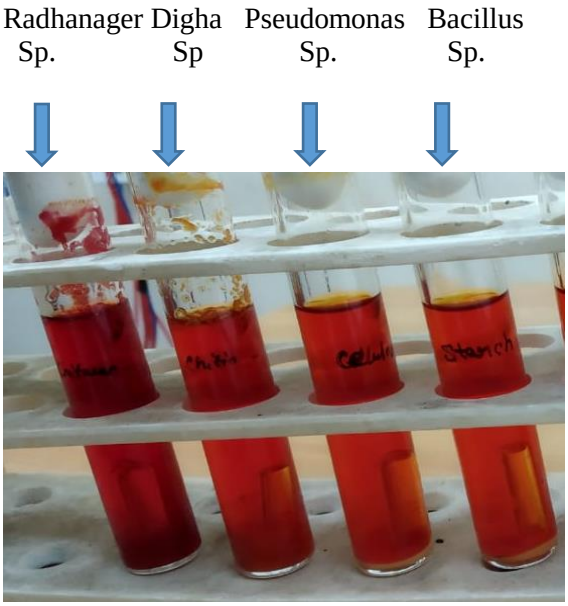


Figure 61: Sucrose present as carbohydrate

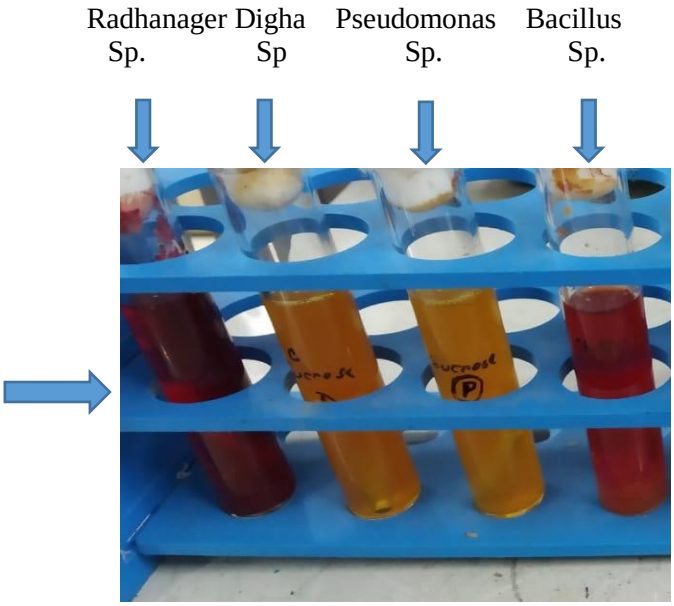


figure 62: Sucrose reduced by the organisms

From the above figure clearly shows that sucrose reduced by the RN bacteria and Pseudomonas species yield produced as color change (red to yellow) due to acid production and bubble indicates the gas production due to oxidation of sucrose.

4.4.5.2 Malonate Utilization Test:

This test detects the ability of an organism to utilize sodium malonate.

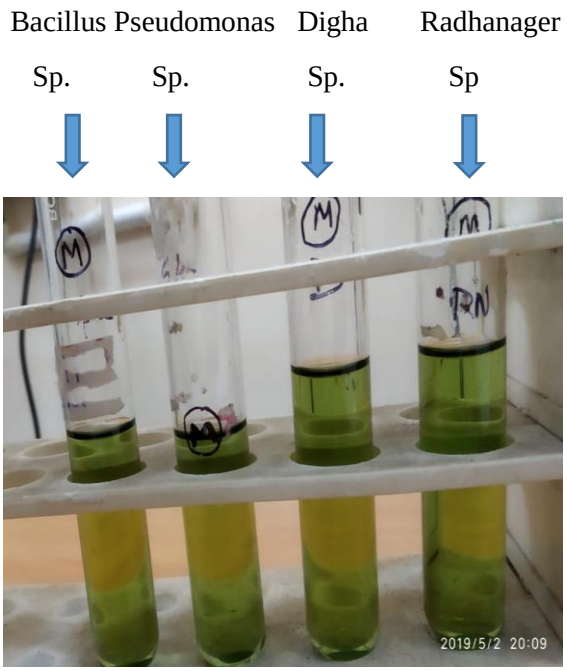


Figure 63: Inoculated bacteria into malonate

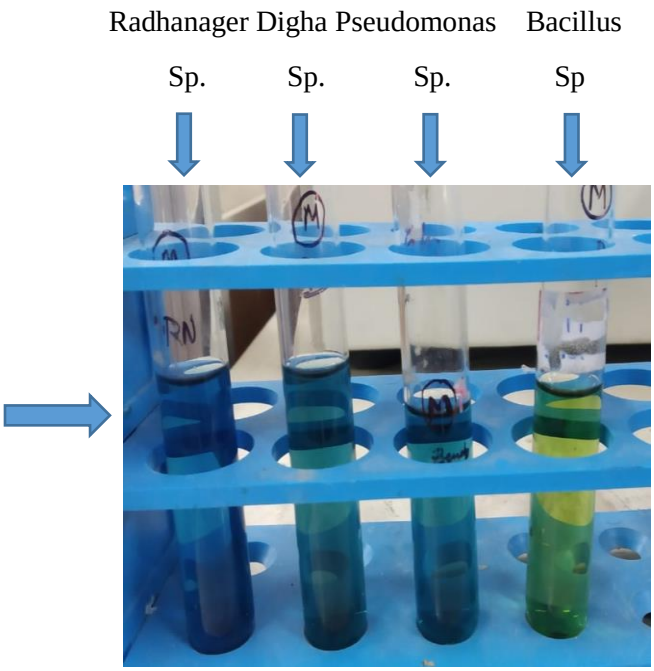


Figure 64: Change in color due to utilization of sodium malonate

From the above figure it is clearly shows that Radhanager sp., Digha Sp. and Pseudomonas can utilize sodium malonate while Bacillus sp. can't utilize malonate.

4.4.5.3 Phenylalanine Deaminase Test:

A small number of bacteria including all the proteae can determinate the aromatic amino acid phenylalanine to produce phenyl pyruvic acid. This end products detected by adding Ferric chloride solution which chelates with the phenyl pyruvic acid to produce a green color. The green fades rapidly so that test must be examined immediately.

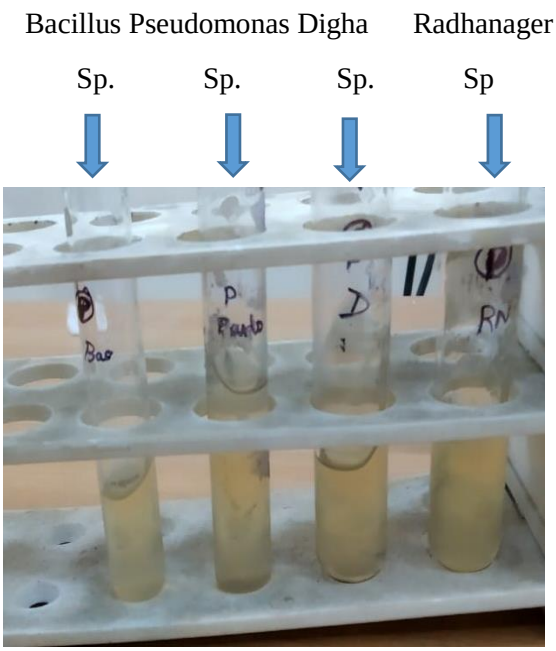


Figure 65: Incubated bacterial slants with ferric Chloride solution

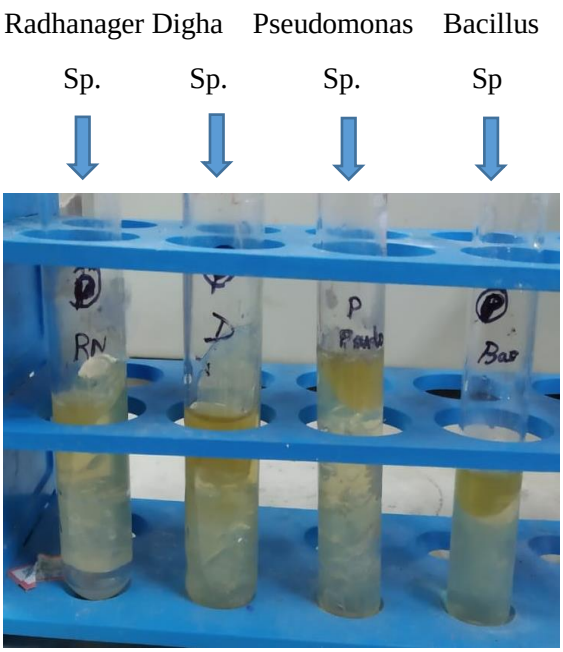


Figure: 66 phenylalanine deasminase positive

From the above figure it is clearly shown that change in the color for all the species indicates phenylalanine deasminase is positive.

4.4.5.4 Starch Hydrolysis Test:

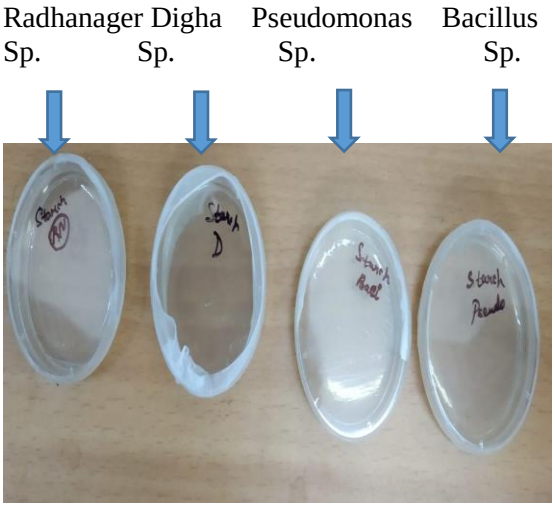


Figure 67: Incubated starch agar plate

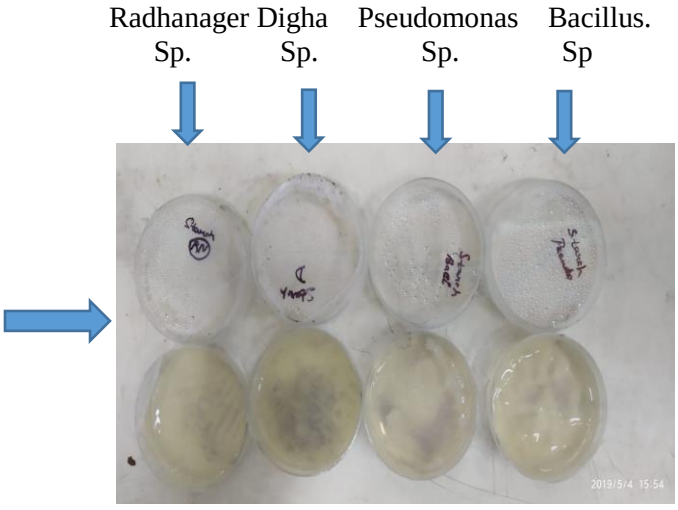


Figure 68 starch agar plates flooded with gram's iodine

Colourless zone surrounding the colonies indicates starch hydrolysis. A blue or purple zone indicate starch is not hydrolysed.

4.4.6 Phenol degradation at variable pH:

In this experimental procedure we examined the phenol degradation with respect to pH variations. Eight setups are made certainly of pH 2, 3, 4, 5.6.7.8 and 9 respectively and two strain strains of microorganisms are tested for the degradation of phenol at different pH. The initial concentration of phenol is taken to be 50 ppm.

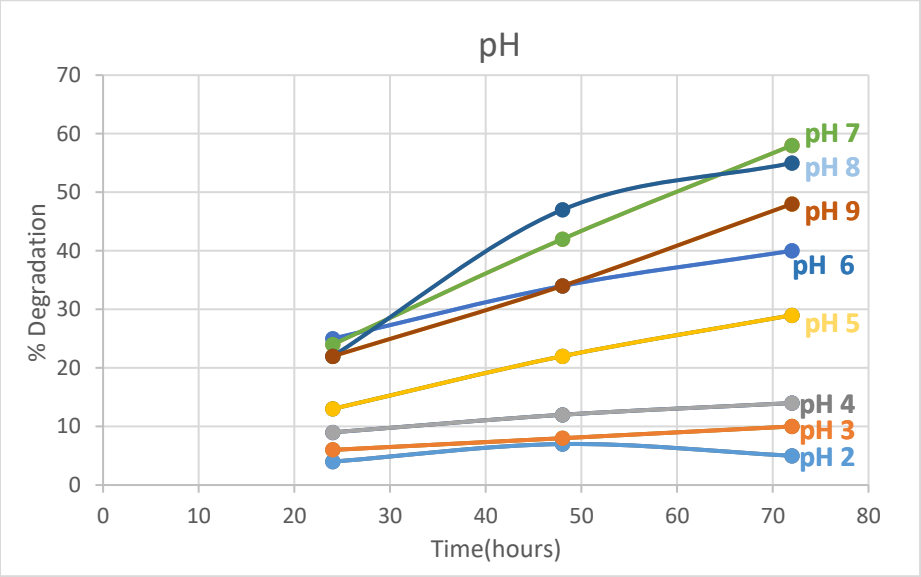


Figure 69 : Phenol degradation growth curve with respect to variable pH for RN

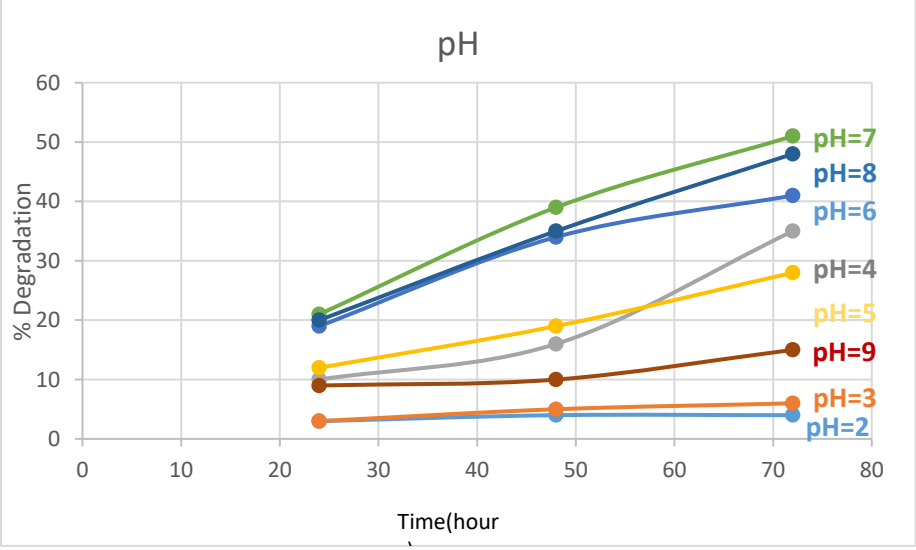


Figure 70: Phenol degradation growth curve with respect to variable pH for D

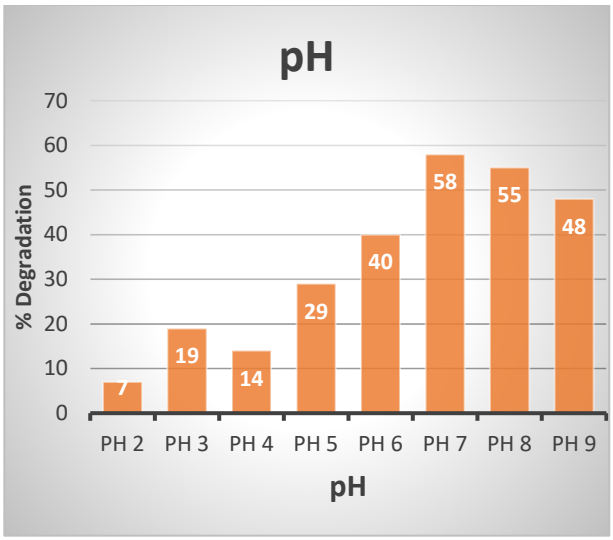


Figure 71: Optimum pH for RN

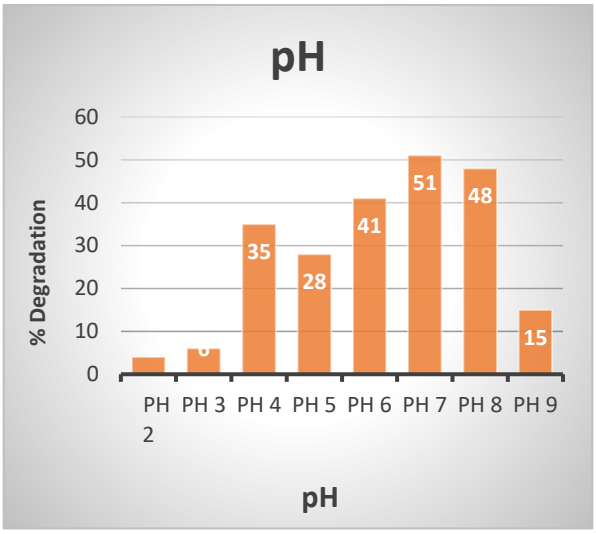


Figure 72: Optimum pH for D

From the above figure maximum degradation of phenol for the RN and D found to be at pH 7.

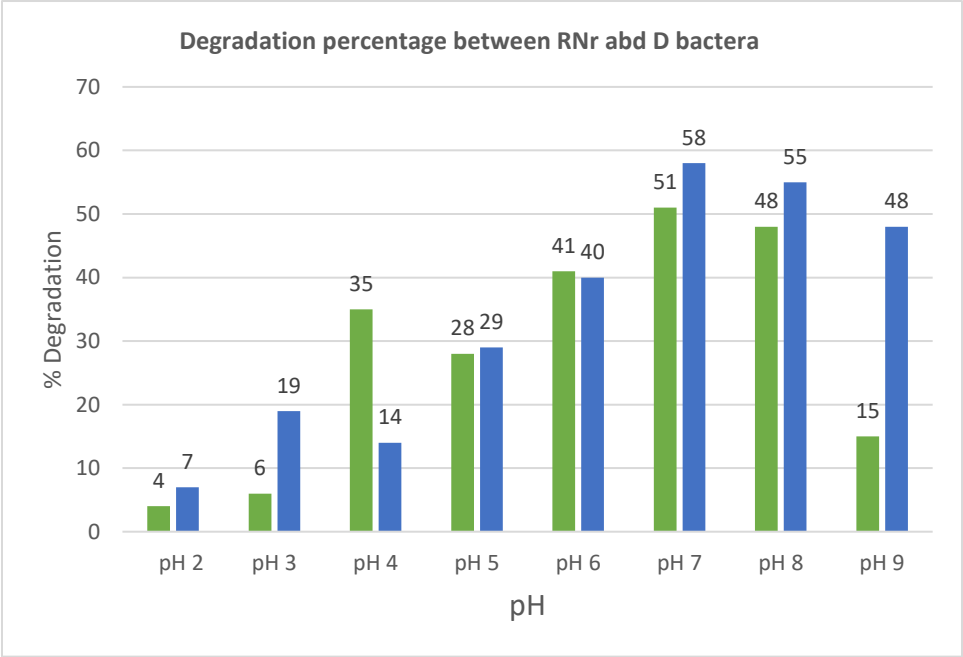


Figure 73: Degradation percentage between RN and D bacteria

4.4.7 Phenol degradation at variable Agitation:

Agitation speed is one of the dependent variable in the optimization process. The experiment were performed in four different variable like stagnant, 80 rpm, 120 rpm .ad 140 rpm at 35°C for 4 days with initial phenol concentration of 50 ppm.

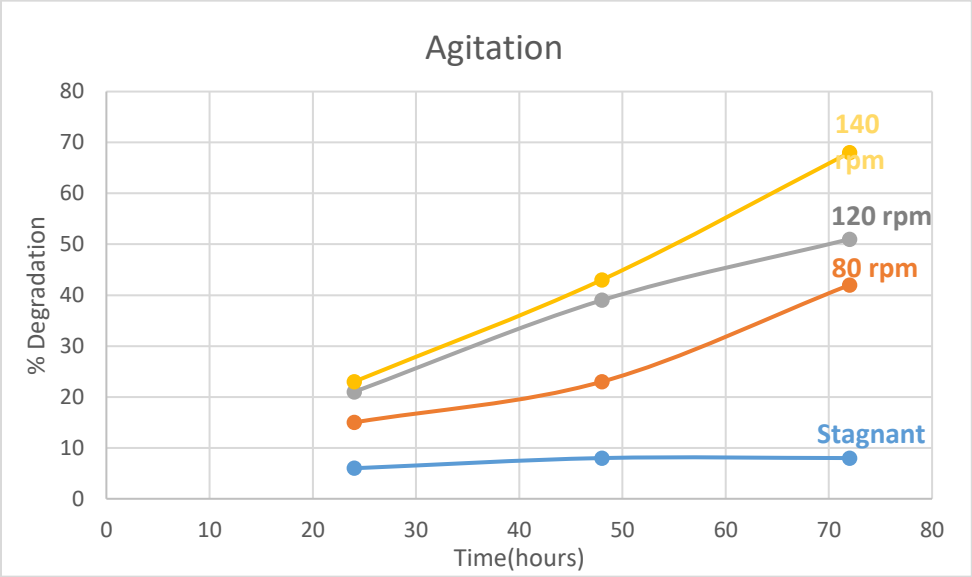


Figure 74: phenol degradation growth curve with respect to variable agitation for RN

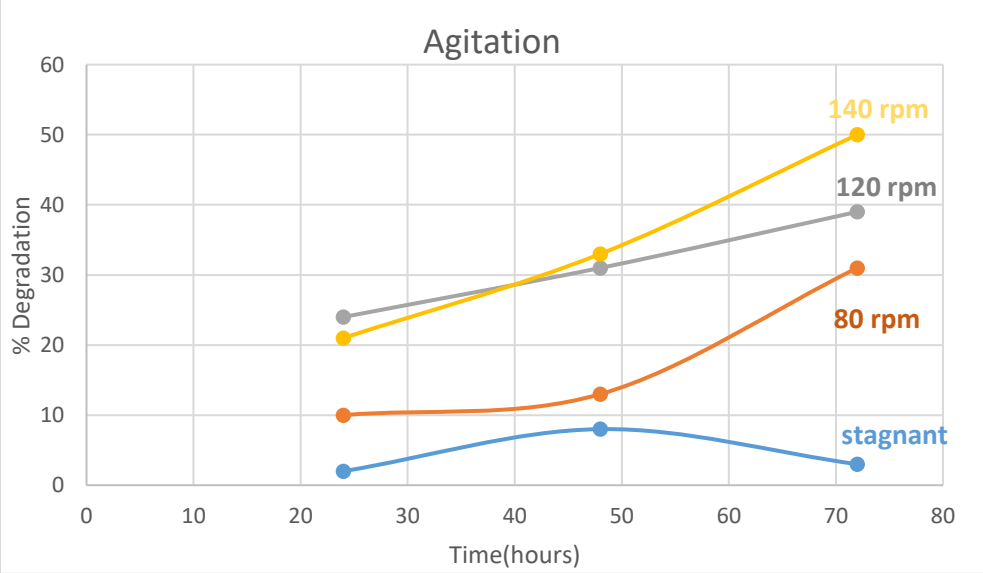


Figure 75: Phenol degradation growth curve with respect to variable agitation for D

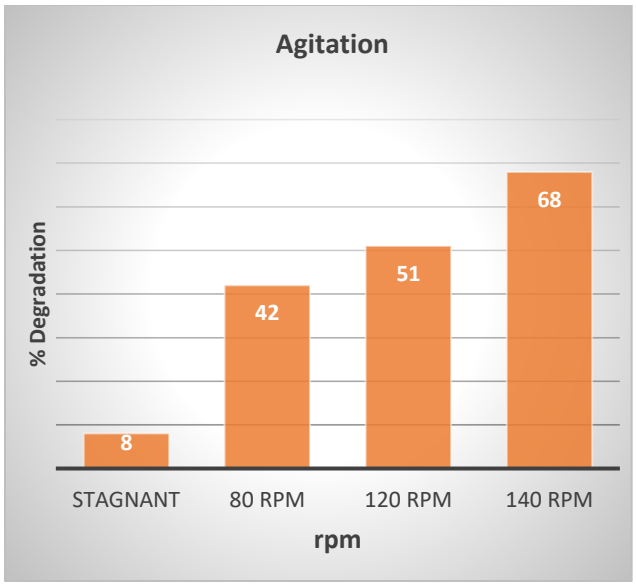


Figure 76: Optimum agitation for Radhanager

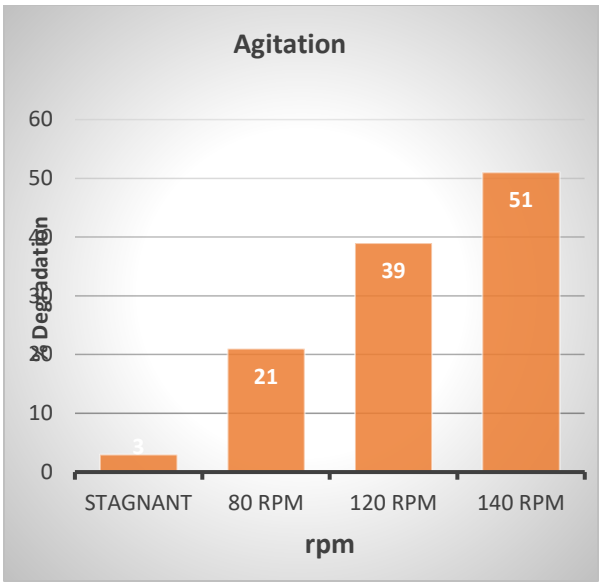
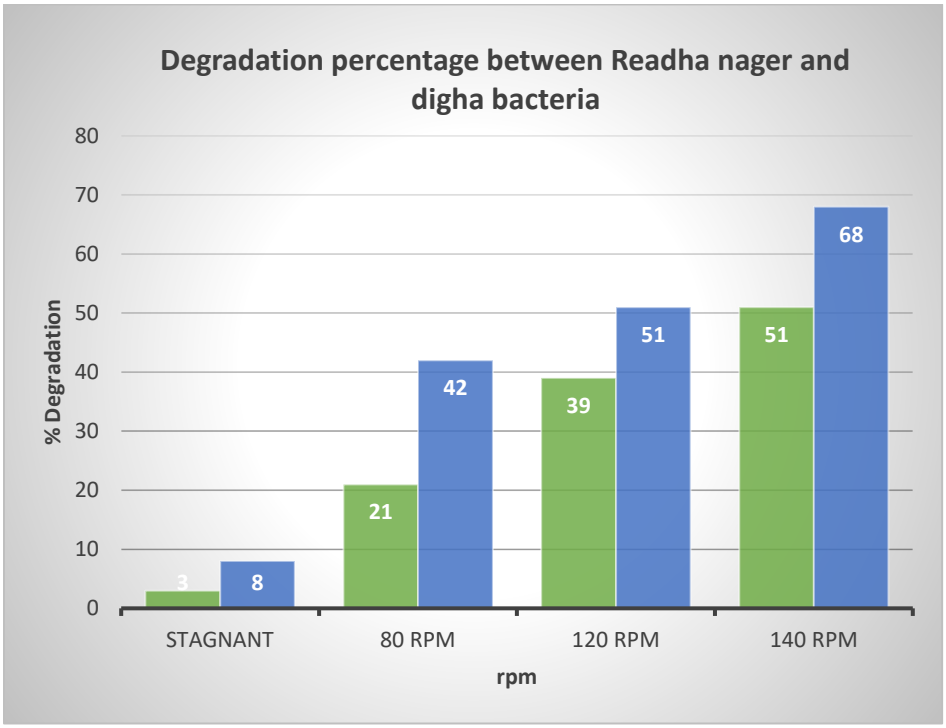


Figure 77: Optimum agitation for Digha



= Radhanager (RN)

= Digha(D)

Figure 78: Degradation percentage between RN and D bacteria

4.4.8 Phenol degradation at variable Temperature:

Temperature is very important factor for degradation in the minimal salt media (MSM) containing phenol. Here we chose three diffent temperature 25°C, 35°C and 45°C with initial phenol concentration of 50 ppm and incubated for three days with agitation of 130 rpm.

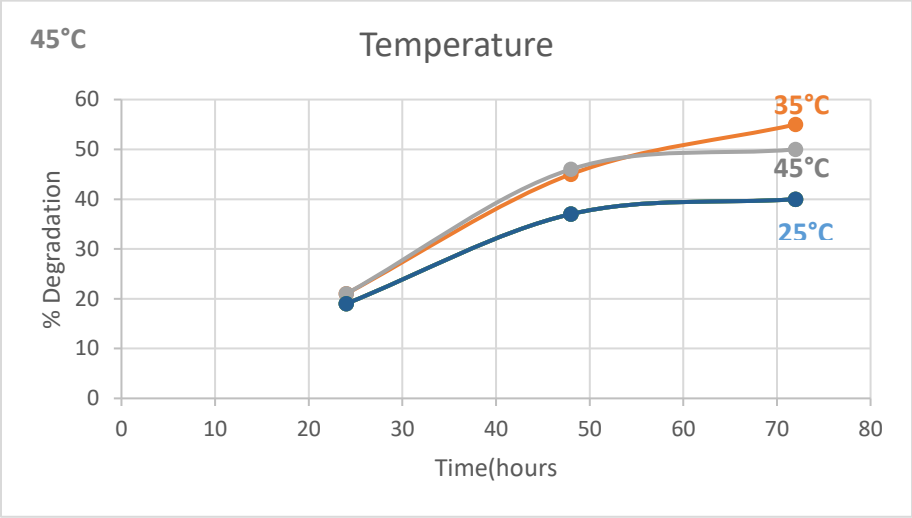


Figure 79: Phenol degradation growth curve with respect to variable temperature for RN

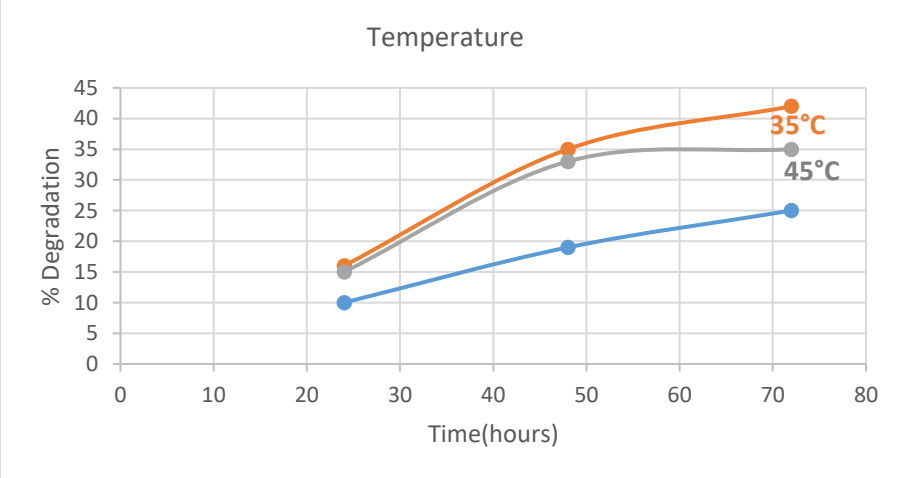


Figure 80 :Phenol degradation growth curve with respect to variable temperature for RN

Optimum temperature for the both the sample is found to be at 35°.

4.4.9 Phenol degradation at variable inoculum size:

Inoculum of different volume were used to inoculate the media was made for the study on the degradation pattern given by inoculum of different sizes with the initial phenol concentration of 50ppm temperature at 35°C, agitation at 130 rpm.

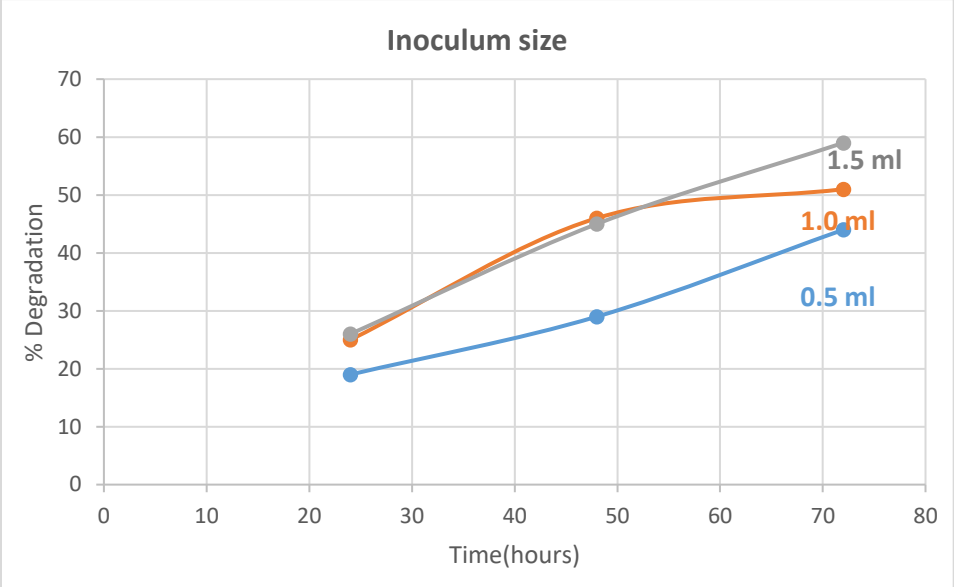


Figure 81: Phenol degradation growth curve with respect to variable inoculum size for RN

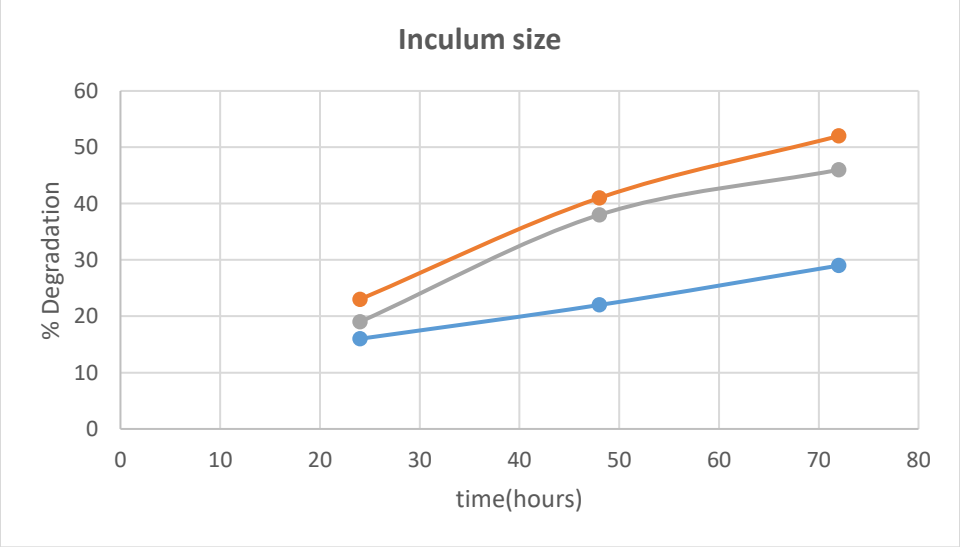


Figure 82: Phenol degradation growth curve with respect to variable inoculum size for D

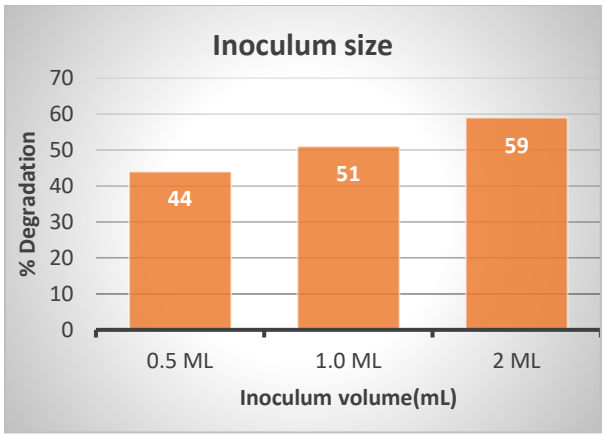


Figure 83: Optimum inoculum size for RN

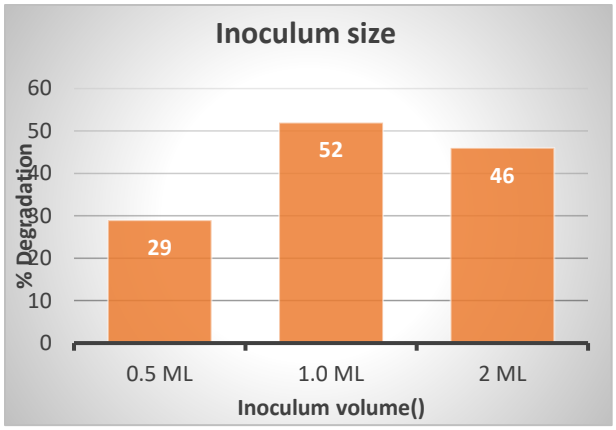


Figure 84: Optimum inoculum size for D

Form the above graph it is clearly shown that the with increase the inoculum size degradation increase at 2ml for the Radhanager while phenol degradation shows it optimal degradation at 1 ml of inoculum size.

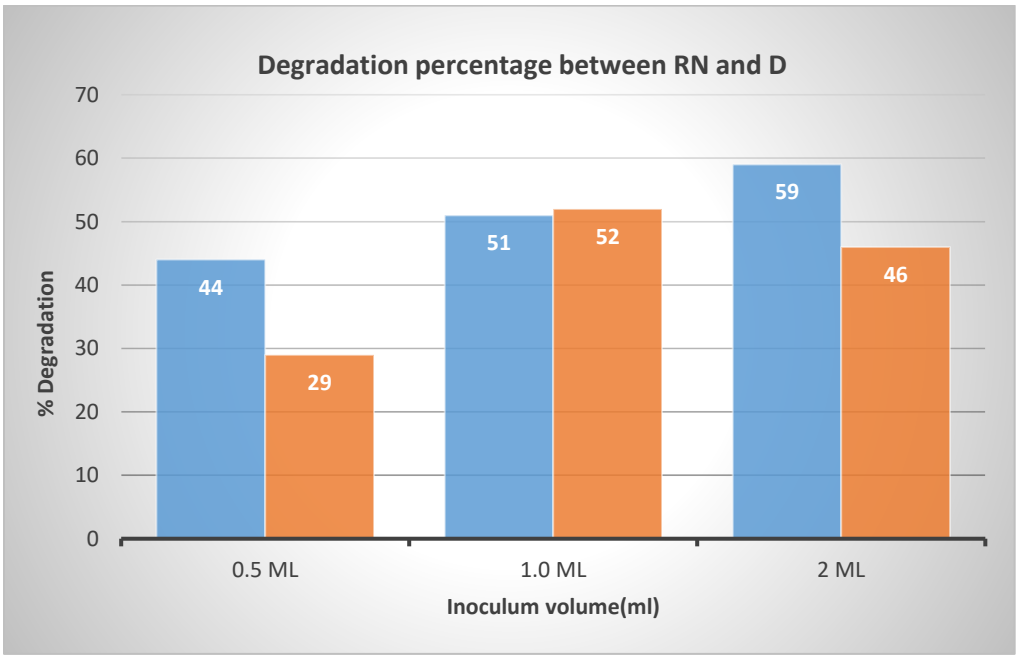


Figure 85: Optimum phenol degradation comparison for sample from RN and D

Chapter 5.

Discussion

In the discussion section first we come across the fungal biodegradation where pre isolated fungi was made to grow on the phenol containing agar plates to check the viability in presence of phenol. . Plates ranging from 50ppm to 500ppm are made to grow in the agar plates by both of the organism i.e. Fungi and bacteria. Selection of phenol tolerating strain is very crucial for the bioremediation study. By similar method bacteria was isolated from marine waters.

pH factor has a crucial role in the growth of organism as some organism are prone to basic pH and some may be acidophilic in nature . This type of organisms are termed as extremophiles. But generally many organisms grows in the neutral zone of pH. They are termed as generalist. In this study fungi shows that their growth and degradation are slightly higher in acidic media of pH ranging from 4 to 6 where it can degrade phenol 70-75% of 100ppm while bacteria shows its optimum phenol degrading capacity at neutral pH with maximum phenol degradation 50-60% of 50ppm.

Temperature plays an important role in the process of biodegradation. As microorganism growth is susceptible to temperature changes thus temperature plays as a key factor in the degradation process of phenol. Fungi and bacteria both shows maximum degradation at optimal temperature of 30°C. At this temperature they can degrade phenol to its maximum potential.

The media containing initial concentration of 100ppm phenol was inoculated by different inoculum volume which affects the overall degradation percentage. Inoculum size of different variable are examined for growth and degradability of phenol at constant temperature and constant phenol concentration for both of the organisms. It is observed that inoculum spore of 10µl shows it maximum degradability for the fungi while 1 ml of bacterial pure culture is optimum for the phenol degradation.

Again, phenol concentration is one of the most important parameter for the degradation for the organisms. Microorganism utilizes the phenol as carbon sauce and utilized phenol as food and hence, the degradation of phenol is observed. It is observed growth of microorganisms inhibited with increasing the concentration off phenol in the media.

Substrate is one of the factor that effects the degradation by introducing substrate such as glucose fructose or sucrose which help microorganism to utilize the substrate of main carbon source which microbes can easily and degrade the phenol hence, affect the growth and degradation.

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Chapter: 6

Conclusion

The results obtained from the above experiments made it clear that phenol is degradable by fungi and bacteria. The experiment emphasizes on the values of variables which plays a key role in the process of biodegradation. Phenol maximum degradation capacities for both fungi is found to be 500ppm which bacteria can degrade phenol up to 300 ppm hence, we can say that fungi is more capable then the bacteria for the degradation.

Maximum degradation for fungi Aspergillus niger is found to be 76% at pH 5 while Alternaria alternata shows it maximum degradation of 56% at pH 6. Also bacteria from Radhanager and Digha shows it maximum degradation at neutral pH.

Various other parameter such as inoculum size, temperature, agitation have been studied. It is investigated the optimum temperature for bacteria and fungi is 35°C and agitation of 140 rpm is more optimum for the degradation of phenol. It is also observed that with the increase in the concentration of phenol to the media growth of the microorganisms inhibited by the phenol concentration

Identification of isolated microorganisms were done by lab scale biochemical methods. Identification of microorganism by nucleotide homology was yet to be done.

We have also optimized the process parameter using Response Surface methodology (RSM) to optimize the process parameter. In conclusion of our experimental work we isolated potent microbial degraders of phenol and optimized its growth conditions.

Chapter: 7

References

Abd-El-Haleem, D., Beshay, U., Abdelhamid, A. O., Moawad, H., & Zaki, S, Effects of mixed nitrogen sources on biodegradation of phenol by immobilized Acinetobacter sp. strain W-17. African Journal of Biotechnology, 2003, 21, 8-12.

Abdelwahab, O., Amin, N. K., & El-Ashtoukhy, E. Z, Electrochemical removal of phenol from oil refinery wastewater. Journal of hazardous materials, 2009, 1632-3, 711-716.

Abuhamed, T., Bayraktar, E , Mehmetoğlu, T., & Mehmetoğlu, Ü, Kinetics model for growth of Pseudomonas putida F1 during benzene, toluene and phenol biodegradation. Process Biochemistry, 2004, 398, 983-988.

Adjei, M. D., & Ohta, Y.. Factors affecting the biodegradation of cyanide by Burkholderia cepacia strain C-3. Journal of bioscience and bioengineering, 2000, 893, 274-277.

Agarry, S. E., Durojaiye, A. O., & Solomon, B. O.. Microbial degradation of phenols: a review. International Journal of Environment and Pollution, 2008, 321, 12-28

Agarry, S. E., Solomon, B. O., & Audu, T. O. K.. Substrate utilization and inhibition kinetics: Batch degradation of phenol by indigenous monoculture of Pseudomonas aeruginosa. International Journal of Biotechnology and Molecular Biology Research, 2010, 12, 22

Agarry, S. E., Solomon, B. O., & Layokun, S. K.. Kinetics of batch microbial degradation of phenols by indigenous binary mixed culture of Pseudomonas aeruginosa and Pseudomonas fluorescence. African Journal of Biotechnology, 2008, 714.

Ahmaruzzaman, M.. Adsorption of phenolic compounds on low-cost adsorbents: a review. Advances in colloid and interface science, 2008, 1431-2, 48-67.

Aiba, S., Shoda, M., & Nagatani, M.. Kinetics of product inhibition in alcohol fermentation. Biotechnology and bioengineering, 1968, 106, 845-864.

Aksu, Z., & Bülbül, G.. Determination of the effective diffusion coefficient of phenol in Ca-alginate-immobilized P. putida beads. Enzyme and microbial technology, 1999, 253-5, 344-348.

Alexander, M.. Biodegradation: problems of molecular recalcitrance and microbial fallibility. In Advances in Applied Microbiology 1965, Vol. 7, pp. 35-80. Academic Press.

Allsop, P. J., Chisti, Y., Moo-Young, M., & Sullivan, G. R.. Dynamics of phenol degradation by Pseudomonas putida. Biotechnology and bioengineering, 1993, 415, 572-580.

Bajaj, M., Gallert, C., & Winter, J.. Biodegradation of high phenol containing synthetic wastewater by an aerobic fixed bed reactor. Bioresource technology, 2008, 9917, 8376-8381.

Bandhyopadhyay, K., Das, D., & Maiti, B. R.. Solid matrix characterization of immobilized Pseudomonas putida MTCC 1194 used for phenol degradation. Applied microbiology and biotechnology, 1999, 516, 891-895.

Bandhyopadhyay, K., Das, D., Bhattacharyya, P., & Maiti, B. R.. Reaction engineering studies on biodegradation of phenol by Pseudomonas putida MTCC 1194 immobilized on calcium alginate. Biochemical Engineering Journal, 2001, 83, 179-186.

Banerjee, A., & Ghoshal, A. K. Isolation and characterization of hyper phenol tolerant Bacillus sp. from oil refinery and exploration sites. Journal of hazardous materials, 2010. , 1761-3, 85-91.

Banerjee, A., & Ghoshal, A. K.. Phenol degradation by Bacillus cereus: pathway and kinetic modeling. Bioresource Technology, 2010, 10114, 5501-5507.

Banerjee, A., & Ghoshal, A. K.. Phenol degradation performance by isolated Bacillus cereus immobilized in alginate. International Biodeterioration & Biodegradation, 2011, 657, 1052-1060.

Bollag, J. M., Shuttleworth, K. L., & Anderson, D. H. Laccase-mediated detoxification of phenolic compounds. Appl. Environ. Microbiol., 1988. ,5412, 3086-3091.

Cromar, N. J., Holmes, M., Fallowfield, H. J., & van den Akker, B.. Application of high rate nitrifying trickling filters for potable water treatment, 2008

Garzillo, A. M. V., Colao, M. C., Caruso, C., Caporale, C., Celletti, D., & Buonocore, V.. Laccase from the white-rot fungus Trametes trogii. Applied microbiology and biotechnology, 1998, 495, 545-551.

Hosseini, S. H., & Borghei, S. M.. The treatment of phenolic wastewater using a moving bed bio-reactor. Process biochemistry, 2005, 403-4, 1027-1031.

Jain, A. K., Gupta, V. K., Jain, S., & Suhas.. Removal of chlorophenols using industrial wastes. Environmental science & technology, 2004, 384, 1195-1200.

Kadhim, H., Graham, C., Barratt, P., Evans, C. S., & Rastall, R. A.. Removal of phenolic compounds in water using Coriolus versicolor grown on wheat bran. Enzyme and Microbial Technology, 1999, 245-6, 303-307.

Léonard, D., & Lindley, N. D.. Growth of Ralstonia eutropha on inhibitory concentrations of phenol: diminished growth can be attributed to hydrophobic perturbation of phenol hydroxylase activity. Enzyme and Microbial Technology, 1999, 253-5, 271-277.

Nair, C. I., Jayachandran, K., & Shashidhar, S.. Biodegradation of phenol. African journal of biotechnology, 2008, 725.

Okeke, B. C., Paterson, A., Smith, J. E., & Watson-Craik, I. A.. The relationship between phenol oxidase activity, soluble protein and ergosterol with growth of Lentinus species in oak sawdust logs. Applied microbiology and biotechnology, 1994, 411, 28-31.

Semple, K. T., & Cain, R. B.. Degradation of phenol and its methylated homologues by Ochromonas danica. FEMS microbiology letters, 1997, 1521, 133-139.

Swan, S. H.. Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. Environmental research, 2008,1082, 177-184.

van Schie, P. M., & Young, L. Y.. Isolation and characterization of phenol-degrading denitrifying bacteria. Appl. Environ. Microbiol., 1998, 647, 2432-2438.

Abd-El-Haleem, D., Beshay, U., Abdelhamid, A.O., Moawad, H., Zaki, H., 2003. Effects of mixed nitrogen sources on biodegradation of phenol by immobilized *Acinetobacter sp.* strain W-17. African Journal of Biotechnology 2(1),

Abuhamed, T., Bayraktar, E., Mehmeto_lu,T., Mehmeto_lu, U., 2004. Kinetics model for the growth of *Pseudomonas putidaF1* during benzene, toluene and phenol biodegradation. Process Biochemistry 39(8),.

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

Agarry, S. E., Solomon, B. O., 2008. Kinetics of batch microbial degradation of phenols by indigenous *Pseudomonas fluorescence.* International Journal of Environmental Science and Technology .

Agarry, S.E., Durojaiye, A.O., Solomon, B.O., 2008. Microbial degradation of phenols: a review. International Journal of Environment and Pollution.

Agency for Toxic Substances and Disease Registry (ATSDR), 2008. Toxicological Profile for Phenol. US department of Health and Human services, ATSDR, US.

Ajay, K.J., Vinod, K.G., Shubhi, J., Suhas, 2004. Removal of chlorophenols using industrial wastes. Environmental Science and Technology

Alexander M., 1965. Biodegradation: Problems of molecular recalcitrance and microbial infallibility. Advances in Applied Microbiology 7, Al-Rekabi, W.S., Qiang, H., Qiang, W.W., 2007. Improvements in Wastewater Treatment Technology. Pakistan Journal of Nutrition .