Major Research Project- Final Report

BIOREMEDIATION TECHNOLOGIES FOR TREATMENT OF PHENOL –STRATEGIES TO ENHANCE PROCESS EFFICIENCY

SUBMITTED BY

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OBJECTIVES

- To isolate, screen and identify phenol-degrading microorganisms from industrial wastewaters.
- Optimization of process parameters.
- Immobilization of organism and the effect of the immobilized organism for the removal of phenol.
- Comparison between free and immobilized organism**.**
- Identification of metabolic pathway of phenol biodegradation.
- To gain a better understanding of the growth and phenol biodegradation kinetics of the microorganism.
- Statistical optimization of medium components and growth conditions by response surface methodology to enhance phenol degradation.
- To study the latest design techniques that include the time saving, cost estimation and optimization

1. INTRODUCTION

With the advent of rapid industrialization, Indian economy had progressed; however people had little awareness that the industrial boon providing employment to a large population would also spread their deadly tentacles and engulf the environment, eventually becoming a major cause of air, water and land pollution. Accordingly, the concerns had been raised by many regulatory bodies including Central Pollution Control Board (India), World Health Organization (WHO), World Bank, Indian Standard Institution, Indian Council of Medical Research, etc and the contribution of industries towards pollution is clearly understood in the recent times. The pollutants from industrial discharge and sewage besides finding their way to surface water reservoirs and rivers are also percolating into ground to pollute ground water sources. The polluted water contributes to undesirable taste, color, odour and most importantly may contain toxic chemicals that may disturb the ecosystem and pose deadly effects on human and animal health. They also subdue plant growth and most annoyingly the toxicants easily enter the food chain. Hence, the past four decades had witnessed a number of treatment technologies to overcome such pollution problems, especially water pollution.

1.1. Xenobiotics:

Organic pollutants comprise a potential group of chemicals which can be dreadfully hazardous to human health. Many of these are resistant to degradation. As they persist in the environment, they are capable of long range transportation, bioaccumulation in human and animal tissue and biomagnification in food chain. (Indu Nair et al., 2008)

Man-made chemicals present in the nature at high concentrations polluting the environment or Chemicals that are foreign to the biosphere are known as xenobioticcompounds. These compounds are not commonly produced by nature. Some microbes havebeen seen to be capable of breaking down of xenobiotics to some extent. But most of the xenobiotic compounds are non-degradable in nature. Such compounds are known to be recalcitrant compounds in nature.

Release of chemical substances due to rapid industrial progress has now become a serious problem causing environmental pollution. Pollutants resembling structural features of xenobiotics mostly include organic sulfonic acids, halogenated aliphatic and polycyclic aromatic hydrocarbons, s-triazines, nitroaromatic compounds, azo compounds and synthetic

polymers. Over the years huge quantity of hazardous waste sites is being generated throughout the world due to accumulation of xenobiotic compounds in soil and water. Polycyclic aromatics, nitroaromatic compounds (NACs), and other hydrocarbons (**PAHs**) constituting crude oil, are among the diverse group of xenobiotic chemicals responsible for immense environmental pollution.

1.2. Industrial Effluents:

Phenolic compounds are natural as well as manmade aromatic compounds. They are associated with pulp mills, coal mines, gasoline, petrochemicals, wood preservation plants, pesticides, insecticides, herbicides, detergents, solvents, polymeric resin production, plastic, rubber-proofing, disinfectants, pharmaceutical, metallurgical, explosive, textile, colour, coffee industries, domestic wastes, agricultural run-off and chemical spills. About 40 percent of globally used colorants contain organically bound chlorine a known carcinogen. All the organic materials present in the wastewater from a textile industry are of great concern in water treatment because they react with many disinfectants especially chlorine. Chemicals evaporate into the air we breathe or are absorbed through our skin and show up as allergic reactions and may cause harm to children even before birth.

1.2.1. Chemicals used in Industries:

Synthetic organic dyes, bleaches and detergents Some chemicals are biodegradable-starch, however others such as dyes are non-biodegradable Thus the effluents could have lower dissolved oxygen concentrations which means higher BOD and COD Solids in textile wastewater come from fibrous substrate and process chemicals, this disturbs the aquatic life by showing oxygen transfer and reducing light penetration.

- **Inorganic chemicals:**

- High concentrations of soluble inorganic salts may make the discharge water stream unsuitable for industrial and municipal use.
- Metals such as chromium and zinc are toxic to aquatic life and should be removed before discharge
- Certain carrier chemicals used in dyeing , such as phenol may add bad taste and odor as well.

1.3. Phenol:

Phenol and its derivatives are common water pollutants and include wide variety of organic chemicals. (Santos et al., 2004). These are aromatic molecules containing hydroxyl group attached to the benzene ring structure. Wastewater with high concentrations of phenol can be treated by physicochemical or biological methods. Phenol, a waste product of industrial processes that is introduced into aquatic ecosystems, adversely affects the indigenous biota, including algae, protozoa, invertebrates, and vertebrates (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). Water pollution by organic and inorganic compounds is of great public concern (Pradeep et al., 2014). Their fate in the environment is of great importance as they are toxic, recalcitrant and bioaccumulating in organisms (Annachatre and Gheewala, 1996).

1.3.1 Chemical structure:

The Figure 1.1 shows, the chemical structure of phenol. Phenol (C_6H_5OH) is the monohydroxy derivative of Benzene and is a clear, colorless-to-white solid, hygroscopic in nature (Srihari.V and Ashutosh Das, 2014). It was first isolated from coal tar in 1834 and was named carbolic acid. It is also called as Benzenol, hydroxybenzene, monophenol, oxybenzene, phenyl alcohol, phenyl hydrate, phenyl hydroxide, phenylic acid, phenylic alcohol. Phenol has a distinct odor that is sweet and tarry. The physical and chemical properties of phenol are listed in Table 1.1.

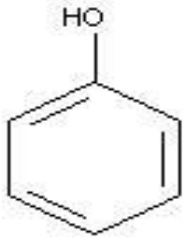

Fig.1.1 Structure of phenol

Table 1.1: Chemical and physical properties of phenol (Kirk-Othmer, 1999)

Property	Phenol
Formula	C_6H_5OH
Molecular weight (g/mol)	94.11
Water solubility (g/L at 25 ºC)	87
Melting point (ºC)	43
Boiling point (ºC)	181.8
Auto ignition temperature	715 ºC
Flash point (open cup)	87 ºC
pKa	9.89×10^{-10}

1.3.2.Sources of phenol:

The origin of phenol in the environment is from natural, man- made and endogenous sources.

- **Natural Sources:**Phenol is a constituent of coal tar, and is formed during decomposition oforganic materials. Natural sources of phenol include forest fire, natural run off from urban area where asphalt is used as the binding material and natural decay of lignocellulosic material.

- **Man made sources:**Phenols are common components of industrial streams, frompolymeric resin producing companies, coal gasification, oil refining, and coking plants. Process industries which are major sources of phenolic discharges are petroleum refineries, gas and coke industries and fibre glass units. Smelting and connected metallurgical operations, plastic industries, pharmaceutical and chemical industries, paint, pulp and paper mills (Kumaran and Paruchuri, 1997), vanish industries and textile units making use of organic dyes also contribute to phenolic liquid waste. (Gurusamy Annaduraia, 2002).

- **Endogenous sources:**An important additional source of phenol may be the formation fromvarious xenobiotics such as benzene (Pekari et al., 1992) under the influence of light.

1.3.3. Phenol as disinfectant:

Disinfectants are antimicrobial agents that are applied to non-living objects to destroy microorganisms that are living on the objects. Disinfectants are different from other antimicrobial agents such as antibiotics, which destroy microorganisms within the body, and antiseptics, which destroy microorganisms on living tissue. Disinfectants are also different from biocides, the latter are intended to destroy all forms of life, not just microorganisms. Disinfectants work by destroying the cell wall of microbes or interfering with the metabolism. Phenolic compounds used as antiseptics or disinfectants include pure phenol and substitution products with halogens and alkyl groups. They act to denature and coagulate proteins and are general protoplasmic poisons.

Phenol (carbolic acid) is one of the oldest antiseptic agents. It is bacteriostatic at concentrations of 0.1%–1% and is bactericidal/ fungicidal at 1%–2%. A 5% solution kills anthrax spores in 48 hr. The bactericidal activity is enhanced by EDTA and warm temperatures; it is decreased by alkaline medium (through ionization), lipids, soaps, and cold temperatures. Concentrations >0.5% exert a local anesthetic effect, whereas a 5% solution is strongly irritating and corrosive to tissues.

Phenol has occupied a prominent place in the field of hospital disinfection since its initial use as a germicide by Lister in his pioneering work on antiseptic surgery. In the past 30 years, however, work has concentrated on the numerous phenol derivatives or phenolics and their antimicrobial properties. Phenol derivatives originate when a functional group (e.g., alkyl, phenyl, benzyl, halogen) replaces one of the hydrogen atoms on the aromatic ring. Two phenol derivatives commonly found as constituents of hospital disinfectants are *ortho*-phenylphenol and *ortho*-benzyl-*para*-chlorophenol. The antimicrobial properties of these compounds and many other phenol derivatives are much improved over those of the parent chemical. Phenolics are absorbed by porous materials, and the residual disinfectant can irritate tissue. In 1970, depigmentation of the skin was reported to be caused by phenolic germicidal detergents containing para-tertiary butyl phenol and para-tertiary amylphenol. Many phenolic germicides are EPA-registered as disinfectants for use on environmental surfaces (e.g., bedside tables, bedrails, and laboratory surfaces) and noncritical medical devices. These are not FDA-cleared as high-level disinfectants for use with semicritical items but could be used to preclean or decontaminate critical and semicritical devices before terminal sterilization or high-level disinfection.

1.3.4. Toxicity of phenol:

There is a long history of human exposure to phenol. Acute exposure of phenol causes central nervous system disorders. It leads to collapse and coma. Muscular convulsions are also noted. A reduction in body temperature is resulted and this is known as hypothermia. Mucus membrane is highly sensitive to the action of phenol. Muscle weakness and tremors are also observed. Acute exposure of phenol can result in myocardial depression.

Phenol causes a burning effect on skin. Whitening and erosion of the skin may also result due to phenol exposure. Phenol has an anaesthetic effect and causes gangrene. Renal damage and salivation may be induced by continuous exposure to phenol. Exposure to phenol may result in irritation of the eye, conjunctional swelling, corneal whitening and finally blindness. Other effects include frothing from nose and mouth followed by headache. Chronic exposure may result in anorexia, dermal rash, dysphasia, gastrointestinal disturbance, vomiting, weakness, weightlessness, muscle pain, hepatic tenderness and nervous disorder. It is also suspected that exposure to phenol may cause paralysis, cancer and gene to fibre striation.

Methemoglobinemia and haemolytic anaemia, as well as liver damage, have also been reported following human exposure to phenol. The odour threshold has been reported to range from 0.021 to 20 mg/m^3 in air, while the threshold for odour in water has been reported to be 7.9 ppm. A taste threshold value of 0.3 ppm water has been suggested. Phenol and its derivatives are toxic and classified as hazardous materials (Zumriye and Gultac, 1999) and it is toxic to both aquatic and terrestrial life (Kanekar et al.,1999). Studies in humans and animals indicate that most of the phenol that enters the body through skin contact, breathing contaminated air, eating food or drinking water, or using products containing phenol, leaves the body in the urine within 24 hours. The normal range of phenol in the urine of unexposed individuals is 0.5-80 milligrams of phenol per litre of urine, and also the toxic levels are different for different organisms as shown in Table 1. 2.

Table 1.2. Toxic levels of phenol in different organisms

Humans:	1 g can be fatal	acc. RIPPEN, 1989
Mammals:		
Rat:	LD_{50} 414-530 mg/kg, oral	acc. RIPPEN, 1989
Rat:	LD_{50} 670 mg/kg, dermal	acc. RIPPEN, 1989

Rabbit:	LD_{50} 400-600 mg/kg, oral	acc. RIPPEN, 1989
Rabbit:	LD_{50} 850 mg/kg, dermal	acc. RIPPEN, 1989
Cat:	LD_{50} 100 mg/kg, oral	acc. RIPPEN, 1989
Dog:	LD_{50} 500 mg/kg, oral	acc. RIPPEN, 1989
Aquatic organisms:		
Pimephales promelas:	LC_{50} 24-68 mg/l	acc. RIPPEN, 1989
Leuciscus idus melanotus:	LC_{50} 25 mg/l (48h)	acc. RIPPEN, 1989
Lepomis macrochirus:	LC_{50} 24 mg/l (96h)	acc. RIPPEN, 1989
Daphnia:	LC_{50} 12 mg/l (48h)	acc. RIPPEN, 1989
Scenedesmus quadricauda:	EC_0 7.5-40 mg/l	acc. RIPPEN, 1989
Microcystis aeruginosa:	EC_0 4.6 mg/l	acc. RIPPEN, 1989

1.3.5. Environmental pollution caused by phenolic waste:

Environmental pollution has been considered as a side effect of various industries. Nowadays, environmental preservation has become a key issue in a society because it is often linked to quality of life. So, increasing awareness on the environment in both developed and developing countries has initiated more studies of possible solutions for controlling pollution. The impacts of pollution on theenvironment have led to an intense scientific investigation.Organic compounds are the major water pollutants, among which phenol andits analogous has been the subject of great concern, as they are toxic in nature andinduce adverse effect on receiving bodies. It is non-persistent in the environment and the major part of phenol in the atmosphere is degraded by photochemical reactions. A minor part will be removed by rain. Phenol in water and soil is degraded by abiotic reactions and microbial activity.They induce genotoxic, carcinogenic, immunogenic, haematological& physiological effects and have a high bioaccumulation rate along the food chain due to its lipophilicity. For example, benzo[*a*]pyrene is recognized as a priority pollutant by the US Environment Protection Agency (Bin Cao et al., 2009).

Thus, phenol is recognized as priority pollutants by "USEnvironmental Protection Agency" (EPA) (1979). The World Health Organization(WHO) recommends the threshold permissible phenolic concentration of $0.001 mgL^{-1}$in portable waters and threshold

concentration of phenol in drinking water below 1.0mg/L. While Ministry of Environment and Forests (MoEF), Government of India,have set a maximum concentration level of 1.0 mgL^{-1} of phenol in the industrialeffluents for safe discharge into surface waters. Thus, the treatment of phenol isnecessitated which is done either by conventional or biological techniques.Favorably, biological degradation is generally preferred as it has advantages of lower costs and possibility of complete mineralization; therefore do not give rise to any hazardous byproducts. Harnessing the potential of microbes to degrade phenol has been an area of considerable study to develop bioremediation approaches which has been considered as a "green option" for treatment of environmental contaminants. (Vojta et al.,2002). The removal of phenol from industrial effluents has attracted researchers from different field. Biological treatment of phenol has therefore been an increasingly important process in pollution prevention. The concentrations of phenol in different indusrial effluents are shown in the Table 1.3.

Table 1.3: Phenol concentrations in industrial effluents (Busca et al., 2008)

Phenol concentration in industrial effluents	**mg L^{-1}**
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
Textile	100-150
Rubber	3-10
Wood preserving industry	50-953

1.4. Applications of phenol:

- **Industrial Use:** Phenol is used in many industries but not restricted to petroleum refineries, gas and coke oven industries, resin manufacturing, tanneries, explosive manufacture, plastic and varnish industries, textile industries, smelting and related metallurgical operations etc. (Mahadevaswamy et al.,1997; Bandyopadhyay et al.,1998; Marrot et al., 2006; Bodalo et al.,2008; Jayachandran and Kunhi, 2008).
- **In hospitals and sanitations:** Phenol has anti-bacterial and anti-fungal properties and hence used in the production of slimicides, disinfectants, antiseptics and medicinal preparations such as ear and nose drops, mouthwashes and sore throat lozenges (ATSDR, 2008).
- **In pharamaceuticals:** Phenol is also a building block for the synthesis of pharmaceuticals (e.g., aspirin) (Busca et al.,2008).
- **Cosmetics:** Phenol is also used in the preparation of cosmetics including sunscreens, hair dyes, and skin lightening products.
- **Agricultural aids:** Phenol is used for the manufacture of herbicides and pesticides.

1.5. Methods involved in the removal of phenolic compounds:

Based on the mode of action, the removal of phenolic compounds is categorized into physical, chemical and biological which is represented in Figure 1.2. Physical methods involve the removal of phenols from aqueous stream, which includes adsorption, solvent extraction, stripping etc. Chemical methods mostly destroy the phenolic compounds either by oxidation or reduction process. Each treatment process and their application are specific to phenol and their substituted phenolic compounds are discussed below.

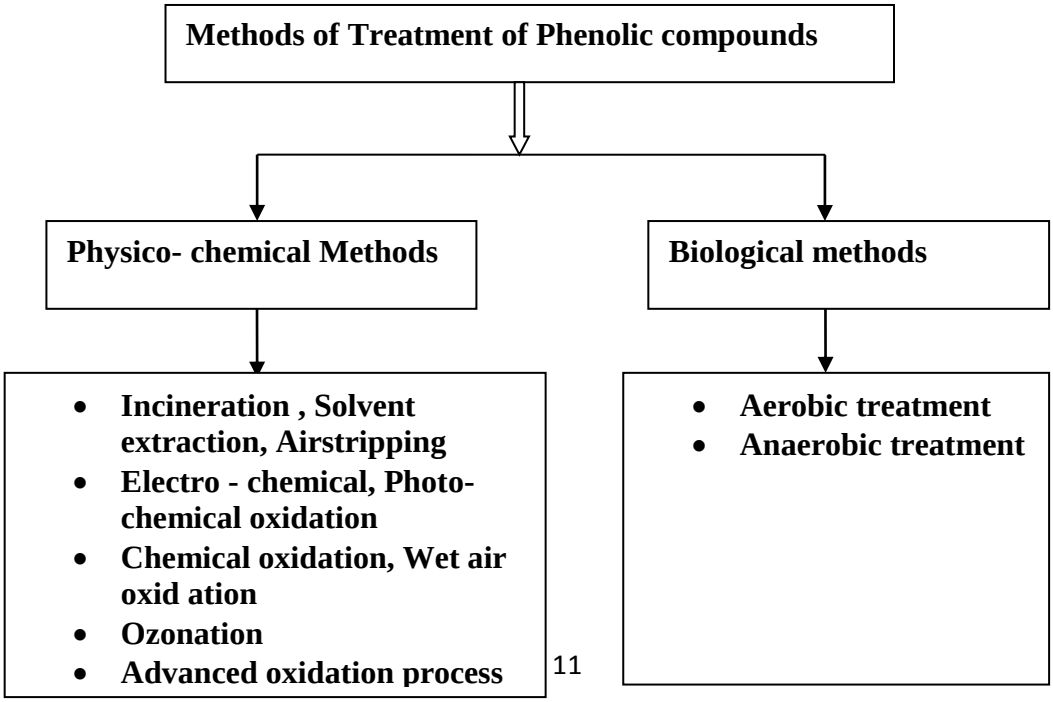

Figure 1.2. Methods involved in the treatment of phenolic compounds

1.5.1. Limitations of the above wastewater treatment techniques:

The physico-chemical treatment technologies 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. 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). 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 the development of technology that emphasizes detoxification and degradation of phenol without the above mentioned drawbacks has become the focus of the research. 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).Phenol biodegradation studies with the bacterial species have resulted in bringing out the possible mechanism and also the enzyme involved in the process. There are reports on many microorganisms capable of degrading phenol through the action of variety of enzymes. Among various enzymes phenol hydroxylase, laccase and catechol 2, 3 dioxygenase are involved more in the biodegradation of phenol (Leonard and Lindley, 1999a; Hublik and Schinner, 2000).

1.6. Biodegradation:

Biodegradation is the breakdown of complex and possibly toxic organic contaminants to non-toxic and simpler elements by microbial activity. These contaminants can be considered as the microbial food source or substrate. Biodegradation of any organic compound 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).

1.6.1. Advantages of biodegradation :

Biodegradation is being preferred over the other conventional treatment methods of phenol due to its potential to degrade phenol completely and overcome the disadvantages posed by other processes .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.

1.7. Microorganisms in biodegradation of phenol:

Environmental biotechnology relies on the pollutant-degrading capacities of naturally occurring microorganisms such as bacteria, fungi and microalgae. Industrial effluents containing different organic and inorganic pollutants require proper treatment prior to discharge into the environments. Among various methods available, biodegradation is environmental friendly and cost effective method. The organic pollutants are used as sole source of carbon and energy for various microorganisms. It is suggested that the microorganisms may adapt themselves to the current environmental conditions by altering

their kinetic parameters and has the ability to adapt to restricted availability of food, depending on the growth conditions of a microorganism. Biodegradation process generally employs microbes like fungi, bacteria etc. Bacteria offers higher advantage over other organisms as it has faster rate of multiplication and they are easier to handle. Hence, there is always a search of bacteria that can degrade phenol effectively and as a consequence of which plenty of literature is available. Many researchers have isolated the bacteria from phenol contaminated site which gives higher probability of acquiring a phenol degrader as the bacteria isolated from such areas usually gets acclimatized to the phenol contaminated environment and may possess excellent machinery to combat higher concentration of phenol and degrade them. Thus, a number of both aerobic and anaerobic phenol degrading microorganisms have been isolated and characterized (Chen et al.,2004; Santos et al.,2001.

A number of microbial species possess enzyme systems that are applicable for the decomposition of various aliphatic and aromatic toxic compounds. Intensive efforts to screen species with high-degradation activity are needed to study their capabilities of degrading phenol and phenolic derivatives. Most of the current research has been directed at the isolation and study of microbial species of potential ecological significance (Krastanov etal.,2013). However, many reports suggest that bacteria degrade phenol through the action of variety of enzymes such as oxygenases hydroxylases, peroxidases, tyrosinases and oxidases. Oxygenases include monoxygenases and dioxygenases. Nonetheless, the mechanism of degradation is generally decided by phenol concentration and nature of the organism of choice.

The mechanism of phenol biodegradation is generally decided by the nature of the organisms, type of the enzyme and the external factors affecting biodegradation. These factors may include temperature, pH, oxygen content and availability, substrate concentration and physical properties of contaminants. Each of these factors should be optimized for the selected organism for the maximum degradation of phenol. When new microorganisms have been isolated with biodegradation efficiency, their biochemical versatility has been found to be immense. However, the isolation of those microbes will often require a targeted intelligent approach to screen the biosphere for its presence (Wackette and Hershberger, 2001). Biotechnology for hazardous waste management involves the development of biological systems that catalyse the detoxification, degradation or decontamination of environmental pollutants. In future technologies, microbial systems might be the potential tools to deal with the environmental pollutants. Biodegradation of phenol by many microorganisms has been studied in order to understand the nutrient requirements, environmental physico-chemical

factors, and complex biochemistry involved that may assist in bioremediation of this toxic compound. Numerous studies have been reported in the literature on the biodegradation of phenol using different microorganisms. Degradation of phenol occurs as a result of the activity of a large number of microorganisms including bacteria, fungi and actinomycetes, Bacterial species include *Bacillus sp*, *Pseudomonas sp*, *Acinetobacte*r *sp*, *Achromobacte*r *sp* etc. *Fusarium sp*, *Phanerocheatechrysosporium*, C*orious versicolo*r, *Ralstonia sp*, *Streptomyces sp* etc are also proved to be efficient fungal groups in phenol biodegradation. Biodegradation of phenol in wastewater is effective in the range of 5-500 mg L^{-1} and higher concentrations slow the growth rate of microorganisms. Concentrations higher than 1450 mg L^{-1} are 11 toxic and kill the entire population of microorganisms in the wastewater (Sevillano et al.,2008). Feitkenhauer et al., (2001) studied the biodegradation of phenol using the thermophile *Bacillus themoleovorans* sp. A2 and reported specific growth rates as high as 2.8 h^{-1} at an initial phenol concentration of 15 mg L^{-1}.

Many studies on biodegradation of phenol come from bacteria. The genus *Pseudomonas* is widely applied for the degradation of phenolic compounds. These bacteria are known for their immense ability to grow on various organic compounds. Phenol biodegradation studies with the bacterial species have resulted in bringing out the possible mechanism and also the enzyme involved in the process. The efficiency of the phenol degradation could be further enhanced by the process of cell immobilization (Annadurai et al., 2000a, b). Phenol and other phenolic compounds are common constituents of many industrial effluents. Once a suitable micro organism based process is developed for the effective degradation of phenol these phenolic effluents can be safely treated and disposed (Borghei and Hosseini, 2004). *Candida tropicalis* RETLCrl from the effluent of the Exxon Mobile Oil Refinery waste water treatment was investigated for phenol degradation using batch and fed batch fermentation under aerobic condition (Mohd Tuah, 2006).

As reported, phenol concentration higher than 1450 mg L^{-1} could not be removed by biological treatment alone. Biological treatments need to be combined with other treatments, such as chemical oxidation, for removing high concentrations of the contaminants effectively and economically from the wastewater. Table 1.4., that reports the various microorganisms involved in phenol degradation

Table 1.4. Microorganisms in phenol degradation

Microorganisms	
Bacteria	**References**
Acinetobacter radioresistens S13	Mazzoli *et al.,* (2007)
Alcaligenes eutrophus	Muller and Babel (1996)
Alcaligenes faecalis	Bai *et al.,* (2007)
Bacillus brevis	Arutchelvan *et al.,* (2005)
Bacillus cereus	Banerjee and Ghoshal (2010)
Bacillus laterosporus	Topalova *et al.,* (1995)
Bacillus stearothermophilus	Mutzel *et al.,* (1996)
Pseudomonas aeruginosa	Jayachandran and Kunhi, (2009**)**
Pseudomonas cepacia	Arutchelvan *et al.,* (2005)
Pseudomonas fluorescens	Viggor *et al.,* (2008)
Pseudomonas pictorium	Annadurai *et al.,* (2000)
Pseudomonas putida	Onysko *et al.,* (2000)
Pseudomonas putida	Viggor *et al.,* (2008)
Fungi	**References**
Aspergillus niger	Garcia *et al.,* (2000)
Aspergillus terreus	Garcia *et al.,* (1997), (2000)
Candida tropicalis	Adav *et al.,* (2007)
Coprinus cinereus	Guiraud *et al.,* (1999)
Coprinus micaceus	Guiraud *et al.,* (1999)
Fusarium flociferum	Cai *et al.,* (2007)
Algae & Cyanobacterium	**References**
Chlorella vulgaris	Shigeoka *et al.,* (1988)
Ochromonas danica	Semple and Cain, (1995), (1996)
Phormidium valderianum BDU30501	Shashirekha *et al.,* (1997)
Selenastrum capricornutum	Shigeoka *et al.,* (1988)

1.8. Factors influencing the degradation of phenolic compounds:

The biodegradation of phenolic compounds relies on the fundamental fact of microbial growth and metabolism. There are factors that can affect degradation ability or metabolism of microorganisms by either preventing or stimulating growth of the organisms and more subtly byaffecting gene expression.

In the natural environment, the rate of degradation can be depends on physical, chemical and biological factors which may differ among ecosystems. Alexander (1994) reported that for a microbial transformation to occur, a number of conditions must be satisfied.

These include:

1. Microorganisms must exist with the required enzyme to catalyze the specific transformation. There are unspecific enzymes that can attack several types of substrates, while other enzymes can only catalyze the breakdown of one specific bond in a specific compound. Duetz et al (1994) reported that different bacterial strains may also degrade same compound by different degradation patterns, depending on the types of enzymes used. Many degradation pathways are achieved only by the synergistic relationship of several species .
2. The chemical must be made available for the microorganism. The inaccessibility may be resulted from the chemical existing in a different phase from the bacteria, for example, in a liquid phase immiscible with water, or sorbed to a solid phase.
3. The success of the degrading strains to proliferate will depend on their ability to compete for the organic compound, oxygen and other environmental factors.
4. The greater the number of substituents in the structure, the more toxic, recalcitrant and less degradable it becomes. For example, substituted phenols such as mono, di-, tri- and penta chlorophenol are more toxic and less degradable than unsubstituted phenol while the position of substituents such as ortho and para-substituted phenols are more degradable than meta-substituted phenols.
5. Environmental factors (Singleton, 1994) such as pH, temperature, nutrient (mineral salt medium), oxygen availability (aeration and agitation), bioavailability and soil type (Talley and Sleeper, 1997).
6. The nutritional requirements of microorganisms normally include nitrogen, phosphorus, potassium, sodium, calcium, magnesium, iron, trace elements and carbon (Oboirien et al., 2005, Ojumu et al., 2005, Yang and Humphrey, 1975).

1.9.Immobilization:

Immobilization is a general term describing a wide variety of the cell or the particle attachment or entrapment (Lopez et al., 1997). It can be applied to basically all types of biocatalysts including enzymes, cellular organelles, animal and plant cells. Currently, different kinds of immobilization have found wide applications not only in the field of biotechnology, but also in pharmaceutical, environmental, food and biosensor industries (Peinado et al., 2005). The cell immobilization emerged as an alternative for enzyme immobilization (Cheetham et al., 1979; Parascandola and Scardi, 1980; Woodward, 1988). Immobilization of cells containing specific enzymes has further advantages such as elimination of long and expensive procedures for enzymes separation and purification and it is vital to expand their application by enabling easy separation and purification of products from reaction mixtures and efficient recovery of catalyst (Junter and Jouene, 2004; Stolarzewicz et al., 2011). In comparison with immobilized enzymes, immobilized cells provide new possibilities since they can be used as natural, water-insoluble carriers of required enzyme activities (Vojtisek and Jirku, 1983). In the case of the immobilization of microbial cells, their field of application spreads from industrial to environmental process. Microorganisms retained on a carrier can be used in continuous and semi-continuous production processes allowing for significant cost decrease, as the biocatalyst does not need to be refilled (Wada et al., 1979; Park and Chang, 2000; Mrudula and Shyam, 2012). Cell immobilization has been defined as the phy-sical confinement or localization of viable microbial cells to a certain defined region of space in such a way as to limit their free migration and exhibit hydrodynamic characteristic which differ from those of the surrounding environment while retaining their catalytic activities for repeated and continuous use (Dervakos and Webb, 1991; Freeman and Lilly, 1998; Covizzi et al., 2007; Amim et al., 2010). Since the early 70s, when Chibata's group announced successful operation of continuous fermentation of Lspartic acid (Coughlan and Kierstan, 1988), numerous research groups have attempted various microbial applications with immobilized cells (Ramakrishna and Prakasham, 1999). Environmental applications of immobilized microbial cells are reported by Bettmann and Rehm (1984), Anselmo et al. (1985), Sahasrabudhe et al. (1988), Oreilly and Crawford (1989), Beunink and Rehm (1990), Balfanz and Rehm (1991), Stormo and Crawford (1992), Cassidy et al. (1996), Wang et al. (1997), Wang et al. (2002), Wang et al. (2007), Zhang et al. (2007), Zhou et al. (2008), Bazot and Lebeau (2009), Wang et al. (2010), Ahmad et al. (2012) and Nickzad et al. (2012). SUPPORT MATERIALS The support selection is one of the crucial decisions to be made in the course of preparation of the immobilization process (Zacheus et al., 2000). For treatment of wastewater, support materials

need to meet the following criteria: insoluble, non-biodegradable, non-toxic, nonpolluting, light weight; flexibility in overall shape, high mechanical and chemical stability, high diffusivity, simple immobilization procedure, high biomass retention, minimal attachment of other organisms and preferably a low cost price (Leenen et al., 1996; Zacheus et al., 2000). Other criteria, such as physical characteristics (porosity, swelling, compression, material and mean particle behavior), as well as, possibility for microbial growth and solubility, are more application specific (Górecka and Jastrzębska, 2011). The carriers are classified as inorganic material (zeolite, clay, anthracite, porous glass, activated charcoal, and ceramics) and organic polymers. Inorganic carriers were selected to immobilize microorganisms because they can resist microbial degradation and are thermostable (Cassidy et al., 1996; Verma et al., 2006). The organic polymeric carriers are more abundant than inorganic carriers and can be natural and synthetic polymeric carriers (Cassidy et al., 1996). Several syn-thetic (acrylamide, polyurethane, polyvinyl, resins) and natural polymer derivatives of algal polysaccharides (alginate, carrageenan, agar, agarose), and chitosan, an amino polysaccharide derived from chitin, has been experimentally used. The most commonly used polymers are the natural polymers alginate and carrageenan but these natural polymers are less stable in wastewater than synthetic polymers (Bashan, 1998; Arica et al., 2004; Moreno-Garrido, 2008; Stolarzewicz et al., 2011). Alginates (polymers made of different proportions and sequences of mannuronic and guluronic acids extracted from brown algae) are easy to handle, nontoxic to humans, the environment, and the entrapped microorganisms, legally safe for human use, available in large quantities, and inexpensive. From a physiological perspective, a major advantage of alginate is that immobilized cells do not suffer extreme changes in physicochemical condition during the procedure of immobilization and the gel is transparent and permeable (Bashan and Bashan, 2010). However, this substance cannot be maintained for a long period in aqueous solution because the encapsulation immobilized microorganism can easily be broken during the operation (Cassidy et al., 1996). Chitosan is inexpensive, non-toxic property and possesses potentially reactive amino functional groups which can enhance the affinity of the carrier with the microorganisms. However, the mechanical stability of the carrier would decrease because of the biodegradability in the course of usage. Other natural gels, such as agar, collagen andagarose, also can be used as microbial encapsulation carriers (Zhou et al., 2008). Some natural polymers are more vulnerable to environmental degra-dation by microbes. However, diffusivity is higher in natural polymers and they are less hazardous to produce (Leenen et al., 1996; Cassidy et al., 1996). Synthetic polymeric supports are not easily biodegradable and have much better mechanical performance

compared with nature carrier. Materials, such as polyacrylamide (PAM), polyvinyl alcohol (PVA), polyethyleneglycol (PEG) and polycarbamoyl sulphonate (PCS) were synthesized as encapsulation carriers (Leenen et al., 1996). In order to improve the stability of gel carrier, various synthetic plastics, for example polypropylene (PP), polyethylene (PE), polyvinylchloride (PVC), poly-urethane (PU) and polyacrylonitrile (PAN) have been explored extensively as immobilized microorganism carriers more recently (Zacheus et al., 2000). Among the various extensively used plastics carriers, polyurethane (PU) is one kind of outstanding carrier for entrapping microorganisms in piloted applications in practical wastewater treatment (Guimarães et al., 2002). Martins et al. (2012) reported potential of the Gramnegative bacterium Serratia marcescens and the yeast Candida rugosa to immobilization on polyurethane foam.

1.10 Methods for Immobilization of microbial cells:

Immobilization of microbial cells in biological processes can occur either as a natural phenomenon or through artificial process (Ramakrishna and Prakasham, 1999). Different immobilization types have been defined: covalent coupling/cross linking, capture behind semipermeable membrane or encapsulation, entrapment and adsorption (Mallick, 2002). The types of immobilization can be grouped as ‘‘passive” (using the natural tendency of microorganisms to attach to surfaces-natural or synthetic, and grow on them) and ‘‘active” (flocculant agents, chemical attachment and gel encapsulation) (Cassidy et al., 1996; Cohen, 2001; Moreno-Garrido, Covalent bonding/Cross linking The mechanism involved in this method is based on covalent bond formation between activated inorganic support and cell in the presence of a binding (crosslinking) agent. For covalent linking, chemical modification of the surface is necessary. Covalent attachment and cross-linking are effective and durable to enzymes, but it is rarely applied for immobilization of cells. It is caused mainly by the fact that agents used for covalent bonds formation are usually cytotoxic and it is difficult to find conditions when cells can be immobilized without any damage (Ramakrishna and Prakasham, 1999). There are few reports of successful covalent binding of the cells and most of them concern yeast. Navarro and Durand (1977) published an article describing a successful way of covalent binding of Saccharomyces carlsbergensis on porous silica beads. Two years later, there was another publication concerning yeast (Saccharmyces cerevisiae, Saccharomyces amurcea) immobilization with this method on borosilicate glass and zirconia ceramics (Messing et al., 1979). Entrapment Entrapment is an irreversible method, where immobilized

cells are entrapped in a support matrix or inside fibers. This technique creates a protective barrier around the immobilized microbes, ensuring their prolonged viability during not only processing but also storage.in polymers (Górecka and Jastrzębska, 2011). Entrapment is the most method extensively studied in cell immobilization. The matrices used are agar, alginate, carrageenan, cellulose and its derivatives, collagen, gelatin, epoxy resin, photo cross-linkable resins, polyacrylamide, polyester, polystyrene and polyurethane (Lopez et al., 1997; Ramakrishna and Prakasham, 1999). Entrapment of the microorganisms in porous polymer carrier was often used to capture the microorganisms from suspended solution and then obtain the immobilized microorganisms. The polymer matrix used in this method confining microorganisms has porous structure, and thus the pollutant and various metabolic products could easily diffuse through into the matrix. In this method, a lot of porous polymers can entrap microorganisms under ambient conditions (Verma et al., 2006). As a rule, the entrapment methods are based on the inclusion of cells within a rigid network to prevent the cells from diffusing into surrounding medium while still allowing penetration of substrate. Entrapment of cells in alginate gel is popular because of the requirement for mild conditions and the simplicity of the used procedure. Several reports are available employing alginate gel (Kierstan and Bucke, 1977). Entrapment allows high mechanical strength, but contains some disadvantages, such as, cell leakage, costs of immobilization, diffusion limitations, and deactivation during immobilization and abrasion of support material during usage. Another disadvantage is low loading capacity as biocatalysts have to be incorporated into the support matrix (Krekeler et al., 1991; Song et al., 2005; Gao et al., 2010; Stolarzewicz et al., 2011). Encapsulation Encapsulation is another irreversible immobilization method, similar to entrapment. In this process, biocatalysts are restricted by the membrane walls (usually in a form of a capsule), but free-floating within the core space (Górecka and Jastrzębska, 2011). The membrane itself is semi-permeable, allowing for free flow of substrates and nutrients (when cells are used as a biocatalyst), yet keeping the biocatalyst inside. The factor determining this phenomenon is the proper pore size of the membrane, attuned to the size of core material. This limited access to the microcapsule interior is one of the main advantages of microencapsulation, for it protects the biocatalyst from the harsh environmental conditions. As most immobilization method, it prevents biocatalyst leakage, increasing the process efficiency as a result (Park and Chang, 2000). The encapsulation method was used to enclose the microorganisms in a polymer-gel by Jen et al. (1996) and is one of the most frequently used in laboratory experiment up to now and there is far away engineering application for wastewater treatment (Lozinsky and Plieva, 1998). However, even though in

encapsulation, high cell loading can be achieved, but the capsules are still very weak (Song et al., 2005). The diffusion limitation is one of the inevitable drawbacks associated with encapsulation method (Lozinsky and Plieva, 1998). Adsorption The immobilization passive or adsorption natural of microorganisms onto porous and inert support materials is similar to the adsorption of colloid particles (Araujo et al., 2010). Apparently, it is the first example of cell immobilization and probably is the simplest method of reversible immobilization (Monsan et al., 1987; Klein and Ziehr, 1990). This technique is based on the physical interaction between the microorganism and the carrier surfaces, while frequently reversible is simple, cheap and effective. The immobilization of microorganisms on properly chosen adsorbents stimulates microbial metabolism, protects cells from unfavorable agents, and preserves their physiological activity (Nikovskaya, 1989; Kozlyak et al., 1991, 1993). Different from the inherent problems associated with cell entrapment, cell immobilization through adsorption provides a direct contact between nutrients and the immobilized cells thus, eliminating such concerns (Braschler et al., 2005). This cell immobilization technique involves the transport of the cells from the bulk phase to the surface of support (porous and inert support materials), followed by the adhesion of cells, and subsequent colonization of the support surface (Kilonzo and Bergougnou, 2012). Adsorption is based on weak forces, however, still enabling an efficient binding process. Usually in bonds formation, several forces are involved: van der Waals forces, ionic and hydrophobic interactions and hydrogen bonds. Both electrostatic and hydrophobic interactions govern the cell-support adhesion, which is the key step in controlling the cell immobilization on the support (Hsu et al., 2004, Górecka and Jastrzębska, 2011). In contrast to ceramics, wood chips and straw, fibrous matrices provide adequate supporting surfaces for cell adsorption (Talabardon et al., 2000; Chu et al., 2009) due to their high specific surface area, void volume, mechanical and permeability, low pressure drop, diffusion problems and toxicity, maximum loading, biodegradability and durability and low cost and high availability (Huang and Yang, 1998). Their natural configuration also allows them to trap more cells than other materials (Yang and Shu, 1996; Yang and Lo, 1998) Polyurethanes foams for immobilization by adsorption Polyurethanes (PU) are one of the most versatile materials in the world today. They are known for being a perfect material for footwear, machinery industry, coatings and paints, rigid insulation, elastic fiber, soft flexible foam, medical devices (Romaškevič et al., 2006). Some time ago PU was found to be applicable in the biochemical and biotechnological fields and flexible polyurethane foams have gained relevance as microbial carriers for their good mechanical properties, high porosity, large adsorption surface, resistance to organic solvents

and microbial attack, easy handling, regenerability and cost effectiveness (Patil et al., 2006). In general, the high rates of sorption of positive charge and hydrophobic character of the polyurethane foam, allow interaction with most microbial cell surfaces (Afghan et al., 1984; Wang et al., 2009). They are inexpensive and easily regenerated by extraction or washing with solvents (Belyakova and Schevchenko, 1986). The microbial immobilization in polyurethane, combined with the use of bioreactors improved significantly the biodegradation process of phenols and derivatives (Pai et al., 1995). The highest efficiency in the degradation of ophthalate by cells Bacillus-spp. immobilized in polyurethane foam, in relation to alginate was reported by Patil et al. (2006). Chanthamalee; Luepromchai (2012) described the efficiency of the Gordonia sp immobilized in polyurethane foam in removing lubricants boats, while Silva et al. (2006) have described that the immobilization of bacteria in polyurethane foam increased resistance to high concentrations of sulphate. Factors affecting microbial cell adsorption there are many factors (such as the age and the physiological state of cells) that influence the sorption of microbial cells. The surface structures of bacterial cells (flagella and other appendages), superficial charges and hydrophobicity also play an important part in the cell adherence to solid surfaces (Donlan, 2002; Chae et al., 2006; Oulahal, et al., 2008). The composition of the medium, its pH, and environmental conditions considerably influence the adsorption of cells by changing their electrokinetic potential (Stanley, 1983; Fletcher and Pringle, 1986; Kilonzo and Bergougnou, 2012). The surface properties of adsorbents also affect the process of cell immobilization (Busalmen and Sanchez, 2001, Ubbink and Schar-Zammaretti, 2007). The degree of cell immobilization depends on the structure and the size of adsorbent pores (Arinbasarova et al., 1982). The nature of adsorbents is also important. Organic adsorbents are chemically stable and show a great variety of surface properties and pore structures, whereas inorganic adsorbents are resistant to biological degradation are affordable, and can be easily regenerated. The disadvantage of inorganic adsorbents is that they are soluble in alkaline solutions (Samonin and Elikova, 2004).

1.11 Gen III Microlog:

GENIII biolog is a software used to identify the microorganism. In GEN III Biolog,s the microplates and database are present these were first introduced in 1989, implement a novel, patented redox chemistry. The GEN III software is used to identify degree of uncertainty for genes and species and it is provide clear indication for genes and species

level identification .The new GENIIIredox chemistry is applicable to the unequalled range of both gram negative and gram positive bacteria. GENIII anatomize and assess the ability of the cell to metabolize all significant Classes of biochemical's, in addition to determining other significant physiological properties such as pH, salt, and lactic acid tolerance, reducing power, and chemical sensitivity. The identification of isolated bacteria was accomplish by new GEN III Microplates™ test panel of the Biolog system. The test panel consist of a "Phenotypic Fingerprint" of the microorganism used to identify them to a species level. The GEN III MicroPlates™ enable testing of Gram-negative and Gram-positive bacteria in the same test panel. The test panel contains 71 carbon sources and 23 chemical sensitivity assays.

Technologies available for biolog include mainly:

Microbial identification technology (species level)

Phenotypic microarray technology (for characterization of microbes)

1.12Polymerase Chain Reaction:

Most phenolic compounds are first con-verted to catechol or protocatechuate.In the _-ketoacidand _-ketoadipate pathways, catechol or protocatechuateis further oxidized by catechol 2,3-dioxygenase, catechol1,2-dioxygenase or protocatechuate 3,4-dioxygenase to _-ketoadipate.This _-ketoadipate is then further converted,with two additional steps, into Krebs cycle intermediates. Many scientists have used the rRNA approach todetect microbial populations and to describe the structures of microbial communities in various environments without isolating the component microorganisms. These studies have shown that most 16S ribosomal DNA (rDNA) sequences directly amplified from environmental samples are different from the sequences of comparable laboratory strains. To better understand phenol we isolated and characterized the phenol-degrading bacteria that were identified by the rRNA approach to be the dominant population in phenol-digesting effluents. Physiological and genetic differences between the dominantphenol-degrading bacteria isolated in this study and representativephenol-degrading bacteria were characterized.

Polymerase Chain Reaction or PCR is a powerful method that allows the species specific detection of organisms in environmental samples based on amplification of DNA specific fragment. Required time for total PCR steps (DNA extraction, PCR protocol and electrophoresis) is one day and it provides higher specificity and sensitivity. Of course the success of PCR for specific detection is highly dependent on the specificity of the nucleotide sequences used as the primers. Pseudomonas species known to be capable of using aromatic

compounds such as phenol as a sole source of carbon and energy. Aerobic biodegradation of phenol starts with oxygenation of phenol by phenol hydroxylase enzymes and the genes coding of these enzymes are used for detection of phenol-degrading bacteria by PCR Biodegradation that exploits the ability of microorganism (generallybacteria) to convert organic pollutants to water, carbon dioxide and biomass under aerobic or anaerobic condition appears to be the most environmentally benign method of removal of pollutants avoiding undesirable by-products or secondary pollutants like in chemical scrubbing or thermal waste gas treatment. Recent studies on the biodegradation of mono-aromatic hydrocarbons such as benzene, toluene and phenol using mixed substrate system revealed a number of substrate interactions during hydrocarbon degradation. Pseudomonas is the most widely reported bacteria for the biodegradation of phenolic compounds and the inhibitory effect of phenol became predominant at higher concentrations.

1.13Growth Kinetics of Pseudomonas syringaepvmaculicola Strain:

Studies have shown that phenol can be aerobically degraded by a wide variety of microorganisms of genera Pseudomonas, Acinetobacter, Alcaligenes, Bacillus,Nocardia, Nocardioides, Ralstonia and Rhodococcus. In this study, we have presented the degradation kinetics of phenol by Pseudomonas. In this article, high initial phenol concentrations were utilized in presence of microorganisms. The kinetic parameters of degradation of phenolwith mixed Pseudomonas strain.

1.14Mathematical Model:

It is necessary to evaluate the relationship between the specific growth rate, μ and the phenol concentration, S. Monod equation is a simple bio kinetic model which was chosen for modelling of two parameters. According to the model, phenol is considered as non-inhibitory compound. Monod's non-inhibitory kinetics equation is presented in the following equation:

$$\frac{dX}{X.dt}$$

Where μ is specific growth rate (h^{-1}), S is limiting substrate concentration;μmax is maximum specific growth rate (h^{-1}), $K_2$is half-saturation coefficient (mg/l). Phenol biodegradation by microbes has generally been known to be inhibited by phenol itself. Hence, Monod equation isunable for describing inhibitory growth of microorganism at higher substrate concentrations. To present the growth kinetics of inhibitory compounds, several kinetics models were fitted to the experimental data for selecting the best models. These models have

been discussed in detail inthe literature by Edwards Haldane's growth model also is selected due to its mathematical simplicity and wide acceptance for representing the growth kinetics of inhibitory substrates. Haldane's inhibitory growth kinetics equation is as follows:

$$\mu = \frac{\mu_{max} s}{K_2 + S}$$

where, K_i is Haldane's growth kinetics inhibition coefficient (mg/l). The specific growth rate (μ) in the exponential phase is calculated using Equation:

where*X*is microorganism concentration (mg/l).

$$\mu = \frac{\mu_{max} s}{K_2 + S + \left(\frac{S^2}{K_i}\right)}$$

2. Statistical design of experiments

2.1. Introduction:

Response Surface Methodology (RSM) is a collection of mathematical and statisticaltechniques for empirical model building. By careful design of experiments, theobjective is to optimize a response (output variable) which is influenced by severalindependent variables (input variables). An experiment is a series of tests, calledruns, in which changes are made in the input variables in order to identify thereasons for changes in the output response (Montgomery, 2005).

For example, the growth of a plant is affected by a certain amount of water x_1 and sunshine x_2. The plant can grow under any combination of treatment x_1 and x_2. Therefore, water and sunshine can vary continuously. When treatments are from a continuous range of values, then a RSM is useful for developing, improving, and optimizing the response variable. In this case, the plant growth y is the response variable, and it is a function of water and sunshine. It can be expressed as

$$y = f(x_1, x_2) + e \tag{2.1}$$

The variables x_1 and x_2 are independent variables where the response y dependson them. The dependent variable y is a function of x_1, x_2, and the experimental error term, denoted as e. The error term e represents any measurement error on the response,as well as other types of variations are not counted in f. It is a statistical error that is assumed to distribute normally with zero mean and variance s^2. In most RSM problems, the trueresponse function f is unknown. In order to develop a proper approximation for f, theexperimenter usually starts with a low-order polynomial in some small region. If theresponse can be defined by a linear function of independent variables, then theapproximating function is a first-order model. A first-order model can be expressed as

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \epsilon \tag{2.2}$$

If there is a curvature in the response surface, then a higher degree polynomial should be used. A second order model can be expressed as

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum \sum_{i<j} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \epsilon \tag{2.3}$$

The β's are a set of unknown parameters. To estimate the values of these parameters, one must collect data on the system they are studying. Because, in general, polynomial

models are linear functions of the unknown β's, we refer to the technique as linear regression analysis.

2.1 The Sequential nature of the Response Surface Methodology:

Most applications of RSM are sequential in nature.

Phase 0: At first some ideas are generated concerning which factors or variables are likely to be important in response surface study. It is usually called a screening experiment. Theobjective of factor screening is to reduce the list of candidate variables to a relatively few sothat subsequent experiments will be more efficient and require fewer runs or tests. Thepurpose of this phase is the identification of the important independent variables.

Phase 1: The experimenter's objective is to determine if the current settings of theindependent variables result in a value of the response that is near the optimum. If the currentsettings or levels of the independent variables are not consistent with optimum performance,then the experimenter must determine a set of adjustments to the process variables that willmove the process toward the optimum. This phase of RSM makes considerable use of thefirst-order model and an optimization technique called the method of steepest ascent(descent).

Phase 2: Phase 2 begins when the process is near the optimum. At this point theexperimenter usually wants a model that will accurately approximate the true responsefunction within a relatively small region around the optimum. Because the true responsesurface usually exhibits curvature near the optimum, a second-order model (or perhaps somehigher-order polynomial) should be used. Once an appropriate approximating model has beenobtained, this model may be analyzed to determine the optimum conditions for the process.This sequential experimental process is usually performed within some region of theindependent variable space called the operability region or experimentation region orregion of interest.

2.2. Design of Experiments (DOE):

DOE analyzes three aspects of the process namely: factors, levels and response. The DOE addresses above three aspects by stipulating the following:

- The factors to be tested
- The levels of those factors
- The structure and layout of experimental runs, or conditions

A well designed experiment is as simple as possible – obtaining the required information in a cost effective and reproducible manner.

2.2.1. Experimental design process:

The flow chart (Figure 2.1) below illustrates the experiment design process

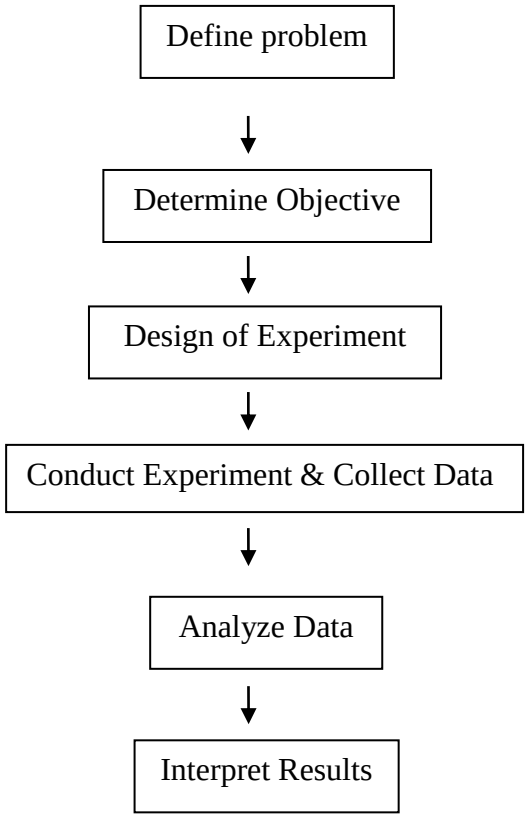

Figure 2.1. Experiment design process

A design is selected based on the experimental objective and the number of factors. The choice of an experimental design depends on the objectives of the experiment and the number of factors to be investigated. Summary table for choosing an experimental design for comparative, screening and response surfaced designs is given in Table 2.1.

Table 2.1 Summary table for choosing an experimental design

Number of factors	Comparative objective	Screening objective	Response surface objective
1	1-factor completely randomized design	-	-
2- 4	Randomized block design	Full or fractional factorial	Central composite or Box-Behnken
5 or more	Randomized block design	Fractional factorial or Plackett- Burman	Screen first to reduce number of factors

2.3. Response surface methodology:

Optimizing refers to improving the performance of a system, a process, or a product in order to obtain the maximum benefit from it. Traditionally, optimization in Bioprocess Engineering has been carried out by monitoring the influence of one factor at a time on an experimental response. While only one parameter is changed, others are kept at a constant level. This optimization technique is called 'one-variable at a time'. The traditional 'one-factor at a time' technique used for optimizing a multivariable system is not only time consuming but also often easily misses the interactions between the components. Also, this method requires carrying out a large number of experiments to determine the optimum levels when the interactions are significant.

In order to overcome this problem, the optimization process has been carried out by using multivariate statistic techniques. Among the most relevant multivariate techniques used in analytical optimization is response surface methodology (RSM). Response surface methodology is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data, which must describe the behavior of a data set with the objective of making statistical previsions. It can be well applied when a response or a set of responses of interest are influenced by several variables. The objective is to simultaneously optimize the levels of these variables to attain the best system performance. Basically, this optimization process involves three major steps: performing the statistically

designed experiments, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of the model.

Before applying the RSM methodology, it is first necessary to choose an experimental design that will define which experiments should be carried out in the experimental region being studied. Several experimental design models exist that reduce the number of experiments and that can be used in different cases. Thus, if it is desired to detect influential factors, experimental designs for first-order models (factorial designs or Plackett–Burman designs) can be used. On the other hand, to approximate a response function or to optimize a process, experimental designs for second-order models should be used, such as three level factorial, Box–Behnken, Central composite, and Doehlert Experimental designs.

2.4. Basic definitions:

Experimental domain is the experimental field that must be investigated. It is defined by the minimum and maximum limits of the experimental variables studied.

Experimental design is a specific set of experiments defined by a matrix composed by the different level combinations of the variables studied.

Independent variables are experimental variables that can be changed independently of each other. Typical independent variables comprise the pH, temperature, carbon source concentration, nitrogen source concentration, inoculum level, rpm, moisture content etc.

Levels of a variable are different values of a variable at which the experiments must be carried out.

Responses or dependent variables are the measured values of the results from experiments.

Residual is the difference between the calculated and experimental result for a determinate set of conditions. A good mathematical model fitted to experimental data must present low residuals values.

$\boldsymbol{R^2}$ - The R^2 (R-square) field contains the coefficient of multiple determinations, which measures the reduction in the total variation of the dependent variable due to the (multiple) independent variables.

Adjusted $\boldsymbol{R^2}$ - The adjusted R^2 is interpreted similarly to the R^2 value except the adjusted R^2 takes into consideration the number of degrees of freedom. It is adjusted by dividing the error sum of squares and total sums of square by their respective degrees of freedom.

Analysis of variance (ANOVA) is a collection of statistical models, and their associated procedures, in which the observed variance is partitioned into components due to different explanatory variables.

2.5. Theory and steps involved in RSM application:

Response surface methodology (RSM) was originated from the graphical perspective generated after fitness of the mathematical models. RSM consists of a group of mathematical and statistical techniques that are based on the fit of empirical models to the experimental data obtained in relation to experimental design. Toward this objective, linear or square polynomial functions are employed to describe the system studied and, consequently, to explore (modeling and displacing) experimental conditions until its optimization.

Some stages in the application of RSM as an optimization technique are as follows: (1) the selection of independent variables of major effects on the system through screening studies and the delimitation of the experimental region, according to the objective of the

study and the experience of the researcher; (2) the choice of the experimental design and carrying out the experiments according to the selected experimental matrix; (3) the mathematic–statistical treatment of the obtained experimental data through the fit of a polynomial function; (4) the evaluation of the model's fitness; (5) the verification of the necessity and possibility of performing a displacement in direction to the optimal region; and (6) obtaining the optimum values for each studied variable.

2.6. Screening of important variables:

Numerous variables may affect the response of the system studied, and it is practically impossible to identify and control the small contributions from each one. Therefore, it is necessary to select those variables with major effects. Screening designs should be carried out to determine which of the several experimental variables and their interactions present more significant effects. Full or fractional two-level factorial designs may be used for this objective principally because they are efficient and economical.

2.7. Choice of the experimental design:

The simplest model which can be used in RSM is based on a linear function. For its application, it is necessary that the responses obtained are well fitted to the following equation:

$$y = \beta_0 \sum_{i=1}^{k} \beta_i x_i + \varepsilon \qquad (2.4a)$$

where*k* is the number of variables, β_0 is the constant term, β_i represents the coefficients of the linear parameters, *x*i represents the variables, and ε is the residual associated to the experiments.

Therefore, the responses should not present any curvature. To evaluate curvature, a second-order model must be used. Two-level factorial designs are used in the estimation of first-order effects, but they fail when additional effects, such as second-order effects, are significant. So, a central point in two-level factorial designs can be used for evaluating curvature. The next level of the polynomial model should contain additional terms, which describe the interaction between the different experimental variables. This way, a model for a second-order interaction presents the following terms:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \qquad (2.4b)$$

where β_{ij} represents the coefficients of the interaction parameters.

In order to determine a critical point (maximum, minimum, or saddle), it is necessary for the polynomial function to contain quadratic terms according to the equation presented below:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \qquad (2.5)$$

where β_{ii} represents the coefficients of the quadratic parameter.

To estimate the parameters in Eq. (2.5), the experimental design has to assure that all studied variables are carried out at in at least three factor levels. Thus, two modeling, symmetrical response surface designs are available. Among the more known second order symmetrical designs are the three-level factorial design, Box–Behnken design, central composite design, and Doehlert design. These symmetrical designs differ from one another with respect to their selection of experimental points, number of levels for variables, and number of runs and blocks.

2.8. Codification of the levels of the variable:

Codification of the levels of the variable consists of transforming each studied real value into coordinates inside a scale with dimensionless values, which must be proportional at its localization in the experimental space. Codification is of concern because it enables the investigation of variables of different orders of magnitude without the greater influencing the evaluation of the lesser.

The following equations can be applied to transform a real value into a coded value according to a determinate experimental design:

For Box -Behnken and Central Composite Designs:

$$x_i = \frac{X_i - \overline{X_i}}{\Delta X_i} \qquad (2.6)$$

where x_i is the coded value of the i^{th}variable, X_i the real value, $\overline{X_i}$ the value at the center point and ΔX_i is the difference between the real value in the central point and the superior level of a variables.

For Doehlert designs:

$$x_i = \left(\frac{X_i - \overline{X_i}}{\Delta X_i} \right) \alpha_i \qquad (2.7)$$

where x_i is the coded value of the i th variable, X_i the real value, $\overline{X_i}$ the value at the center point and ΔX_i is the step change value, and α_i is the maximum value of the coded factor (i.e. 1.0, 0.866 and 0.816 for five levels, seven levels and three levels, respectively).

2.9. Box–Behnken design:

Box and Behnken suggested how to select points from the three-level factorial arrangement, which allows the efficient estimation of the first- and second-order coefficients of the mathematical model. These designs are, in this way, more efficient and economical then their corresponding 3*k* designs, mainly for a large number of variables.

In Box–Behnken designs, the experimental points are located on a hyper sphere equidistant from the central point, as exemplified for a three-factor design. Its principal characteristics are:

1. It requires an experiment number according to $N = 2k(k-1) + cp$, where k is the number of factors and (cp) is the number of the central points;
2. All factor levels have to be adjusted only at three levels (−1, 0, +1) with equally spaced intervals between these levels.

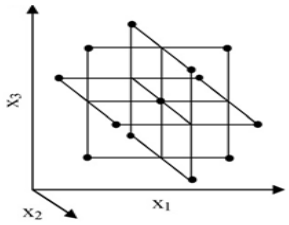

Fig.2.1 Box–Behnken design for the optimization of three variables

Fig. 2.1 presents the Box–Behnken design for three-variable optimization with its 13 experimental points.

This experimental design has been applied for the optimization of several chemical and physical processes; however, its application in analytical chemistry is still much smaller in comparison with central composite design.

2.10. Analysis of a Second-Order Response Surface:

When the experimenter is relatively close to the optimum, a model that incorporates curvature is usually required to approximate the response. In most cases the second order model

$$y = \beta_0 + \sum^{k}_{i=1} \beta_i x_i + \sum^{k}_{i=1} \beta_{ii} x_i^2 + \sum\sum^{k}_{i<j} \beta_{ij} x_i x_j + \xi \qquad (2.8)$$

is adequate. In this section we will show how to use this fitted model to find the optimum set of operating conditions for the x' s and to characterize the nature of the response surface.

2.11. Location of the Stationary Point:

Suppose we wish to find the levels of $x_1, x_2, - - - -, x_k$ that optimize the predicted response. This point, if it exists, will be set of $x_1, x_2, - - - -, x_k$ for which the partial derivatives $\partial y/\partial x_1 = \partial y/\partial x_2 = - - - = \partial y/\partial x_k = 0$. This point, say $x_{1,s}, x_{2,s}, - - - -, x_{k,s}$, is called the stationary point. The stationary point could represent (1) a point of maximum response, (2) a point of minimum response, or (3) a saddle point.

Contour plots play a very important role in the study of the response surface. By generating contour plots using computer software for response surface analysis, the experimenter can usually characterize the shape of the surface and locate the optimum with reasonable precision.

We may obtain a general mathematical solution for the location of the stationary point. Writing the second-order model in matrix notation, we have

$$y = \beta_0 + \mathbf{x'\,b + x'\,Bx} \qquad (2.9)$$

where, **b** is a ($k\times$ 1) vector of the first-order regression coefficients and **B** is a ($k \times k$) symmetric matrix whose main diagonal elements are the pure quadratic coefficients (β_{ij}) and whose off-diagonal elements are one-half the mixed quadratic coefficients (β_{ij}, $i \neq j$). The derivative of y with respect to the elements of the vector x equated to **0** is

$$\partial y/\partial x_1 = \mathbf{b} + 2\mathbf{Bx} = 0 \qquad (2.10)$$

The stationary point is the solution to equation 2.10, or

$$\mathbf{x_s = -B^{-1}}b/2 \qquad (2.11)$$

Furthermore, by substituting equation 2.11 into equation 2.9, we can find the predicted response at the stationary point as

$$Y_s = \beta_0 + x'_s b/2 \qquad (2.12)$$

The eigen values of matrix **B** give an indication about the nature of response surface. For a function representing maximum surface, all the eigen values are negative, whereas they are positive for a function with minimum. If eigen values have different signs (i.e. some positive,

and some negative), the surface is saddle for which a minimum or maximum cannot be specified.

3. REVIEW OF LITERATURE

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.

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). One adaptive mechanism enabling several *Pseudomonas* strains to grow in the presence of membrane-disrupting compounds is the isomerization of *cis*unsaturated fatty acids to *trans*-unsaturated fatty acids. This mechanism could also be found in *Pseudomonas* sp. Strain ADP.

Biodegradation of a substrate by a microorganism depends on a number of factors and it is quite essential to understand how those factors affect the degradation profile of the microbe. Selecting the correct physiological conditions is always a major concern as traditional experiment design would require many experimental runs to achieve satisfactory result.

An adequate number of reports are available on the phenol degrading microorganisms, isolation and optimization of parameters.

Mutzel*et al.*, (1996) isolated a phenol-degrading thermophilic bacterium, designated *Bacillus* sp. A2, from a water and mud sample from a hot spring in Iceland. The aerobic isolate grew optimally on phenol at 65 °C. At 70 °C, 85% of the optimal growth rate was still observed. No growth was observed at 40 °C and 75 °C. *Bacillus* sp. A2 is a gram-positive spore-forming rod. According to 16S rDNA analysis *Bacillus* sp. A2 is closely related to *Bacillus stearothermophilus, Bacillus kaustophilus* and *Bacillus thermoleovorans*. *Bacillus* sp. A2 degraded phenol completely in concentrations up to 5 mM. In addition, all three isomers of cresol were utilized as sole carbon and energy sources. The degradation of phenols proceeds via the *meta*-cleavage pathway and the enzymes involved in its degradation are constitutively expressed.

Semple *et al.*, (1996) reported that the eukaryotic algae *Ochromonas danica*, a nutritionally versatile, mixotrophic chrysophyte, grew on phenol as the sole carbon source in axenic culture and removed the phenol carbon from the growth medium. Respirometric studies confirmed that the enzymes involved in phenol catabolism were inducible and that the alga oxidized phenol; the amount of oxygen consumed per mole of oxidized substrate was approximately 65% of the theoretical value. [U-14C] phenol was completely mineralized,

with 65% of the 14C label appearing as $14CO_2$, approximately 15% remaining in the aqueous medium, and the rest accounted for in the biomass. Analysis of the biomass showed that 14C label had been incorporated into the protein, nucleic acid, and lipid fractions; phenol carbon is thus unequivocally assimilated by the alga. Phenol-grown cultures of *O. danica* converted phenols to the corresponding catechols, which were further metabolized by the meta-cleavage pathway. This surprising result was rigorously confirmed by taking the working stock culture through a variety of procedures to check that it was axenic and repeating the experiments with algal extracts. This is, as far as is known, the first definitive identification of the meta-cleavage pathway for aromatic ring degradation in a eukaryotic alga, though its incidence in other eukaryotes has been (infrequently) suggested.

Shashirekha *et al.*, (1997) concluded that the phenol, the toxic constituent of several industrial effluents, was found to be effectively removed and degraded by the marine cyanobacterium *Phormidium valderianum BDU 30501*. The organism was able to tolerate and grow at a phenol concentration of 50 mg L^{-1} and remove 38 mg L^{-1} within a retention period of 7 days. The removal and degradation were confirmed by changes in the ultraviolet absorption spectra in the culture filtrate, colorimetric estimation of residual phenol and measuring the intracellular activity of the inducible polyphenol oxidase and laccase enzymes. This opens up the possibility of treating a variety of phenol-containing industrial effluents using this organism.

Van Schie *et al.*, (1998) isolated and characterized three novel nitrate-reducing microorganisms that are capable of using phenol as a sole source of carbon from anaerobic sediments obtained from three different geographic locations. The three strains were shown to be different from each other based on physiologic and metabolic properties. Even though analysis of membrane fatty acids did not result in identification of the organisms, the fatty acid profiles were found to be similar to those of *Azoarcus* species. Sequence analysis of 16S ribosomal DNA also indicated that the phenol-degrading isolates were closely related to members of the genus *Azoarcus*. The results of this study add three new members to the genus *Azoarcus*, which previously comprised only nitrogen-fixing species associated with plant roots and denitrifying toluene degraders.

Balan *et al.*, (1999) used *Pseudomonas pictorum* (NICM-2077), an effective strain in the biodegradation of phenol. The microorganism was grown on various nutrient compounds which protect it while confronting shock loads of concentrated toxic pollutants during waste

water treatment. The effect of glucose, yeast extract, $(NH_4)_2SO_4$ and NaCl on phenol degradation has been investigated and an Artificial Neural Network (ANN) Model has been developed to predict degradation. The network model was then compared with a Multiple Regression Analysis model (MRA) arrived from the same training data.

Guiraud*et al.*, (1999) studied on three species of *Coprinus*: *C. sp, C. cinereus and C. Micaceus,* compared on solid media for some aspects of their physiological behaviour and cultural requirements (temperature, pH, substrate). Constitutive extracellular enzymatic activities were also determined. The *Coprinus spp*. exhibited different physiological and cultural features. Cultures of the 3 *Coprinus* species in synthetic liquid medium showed an efficient degradation of phenolic lignin model compounds (catechol, ferulic acid, guaiacol, phenol, protocatechuic acid syringic acid and vanillic acid) and pentachloronitrobenzene, while pentachlorophenol was not metabolized after 5 days perhaps because of a strong adsorption on mycelial biomass. It was suggested that phenoloxidases were not necessarily required for the metabolization of these compounds. *Coprinus* species may share a common degrading system for monomeric phenolic and chloroaromatic compounds.

Artur Eduardo Ribeiro Bastos *et al.*,(2000) isolated two phenol-degrading microorganisms from Amazonian rain forest soil samples after enrichment in the presence of phenol and a high salt concentration. The yeast *Candida tropicalis* and the bacterium *Alcaligenes faecalis* were identified using several techniques, including staining, morphological observation and biochemical tests, fatty acid profiles and 16S/18S rRNA sequencing. Both isolates, *A. faecalis* and *C. tropicalis*, were used in phenol degradation assays, with *Rhodococcus erythropolis* as a reference phenol-degrading bacterium, and compared to microbial populations from wastewater samples collected from phenol-contaminated environments. *C. tropicalis* tolerated higher concentrations of phenol and salt (16 mM and 15%, respectively) than *A. faecalis* (12 mM and 5.6%). The yeast also tolerated a wider pH range (3–9) during phenol degradation than *A. faecalis* (pH 7–9). Phenol degradation was repressed in *C. tropicalis* by acetate and glucose, but not by lactate. Glucose and acetate had little effect, while lactate stimulated phenol degradation in *A. faecalis*. They reported that these soils had never been contaminated with man-made phenolic compounds and this is the first report of phenol-degrading microorganisms from Amazonian forest soil samples. The results support the idea that natural uncontaminated environments contain sufficient genetic diversity to make them valid choices for the isolation of microorganisms useful in bioremediation.

Heinaru *et al.*, (2000) isolated and characterised a total of 39 phenol- and p-cresol-degraders from the river water continuously polluted with phenolic compounds of oil shale leachate. The Species identification done by BIOLOG GN analysis revealed 21 strains of *Pseudomonas fluorescens* (4, 8 and 9 of biotypes A, C and G, respectively), 12 of *Pseudomonas mendocina,* four of *Pseudomonas putida* biotype A1, one of *Pseudomonas corrugata* and one of *Acinetobacter* genospecies 15. Computer-assisted analysis of rep-PCR fingerprints clustered the strains into groups with good concordance with the BIOLOG GN data. Three main catabolic types of degradation of phenol and p-cresol were revealed. Type I, or meta-meta type (15 strains), was characterized by meta cleavage of catechol by catechol 2,3-dioxygenase ($C_{23}O$) during the growth on phenol and p-cresol. These strains carried C23O genes which gave PCR products with specific xylE-gene primers. Type II, or ortho-ortho type (13 strains), was characterized by the degradation of phenol through ortho fission of catechol by catechol 1,2-dioxygenase ($C_{12}O$) and p-cresol via ortho cleavage of protocatechuic acid by protocatechuate 3,4-dioxygenase ($PC_{34}O$). These strains carried phenol monooxygenase gene which gave PCR products with pheA-gene primers. Type III, or meta-ortho type (11 strains), was characterized by the degradation of phenol by $C_{23}O$ and p-cresol via the protocatechuate ortho pathway by the induction of $PC_{34}O$ and this carried $C_{23}O$ genes which gave PCR products with $C_{23}O$-gene primers, but not with specific xylE-gene primers. In type III strains phenol also induced the p-cresol protocatechuate pathway, as revealed by the induction of p-cresol methylhydroxylase. These results demonstrate multiplicity of catabolic types of degradation of phenol and p-cresol and the existence of characteristic assemblages of species and specific genotypes among the strains isolated from the polluted river water.

Onysko *et al.*,(2000) determined the effect of temperature on cell growth and phenol biodegradation kinetics of the psychrotrophic bacterium *Pseudomonas putida* Q5 using both batch and continuous cultures in the range of 10-25 degrees C. The Haldane equation was found to be the most suitable substrate-inhibition model for the specific growth rate. The Haldane parameters mu(max) and K(I) were best modelled by a square-root dependency on temperature. However, the Arrhenius model provided a better prediction of the temperature dependence of K(S). The variation of the yield constant with temperature also was studied experimentally.

Feitkenhauer *et al.* (2001) studied the effect of pH on the phenol biodegradation by the thermophilic strain *Bacillus thermoleovorans* sp A2 and found that highest growth rate was at pH 6.0. Effect of physiological factors like pH, temperature, concentrations of glucose and ammonium sulfate on the biodegradation potential of *Pseudomonas putida* (ATCC 3180) was studied by Annadurai et al. (2002). They reported that the optimum values for maximum degradation of phenol were pH 7.0, temperature 300C, glucose 0.6 g/l and ammonium sulfate 0.6 g/l.

Wael *et al.*, (2003) isolated new phenol degrading bacteria with high tolerance and high biodegradation activity. The isolates were *Burkholderia capacia* PW3 and *Pseudomonas aeroginosa* AT2. Both the isolates could grow aerobically on phenol as sole carbon source and tolerated up to 3000ppm of phenol. The metabolic pathway for phenol biodegradation in both the strains was assigned to the meta-cleavage activity of catechol 2,3-dioxygenase.

Jiang *et al.*, (2004b) isolated 10 bacterial strains from their aerobic phenol degrading granules, identified their potential for degrading phenol. physiological characteristics of ten bacterial strains isolated from phenol-degrading aerobic granules were evaluated in order to identify competitive traits for dominant growth in aerobic granules. The ten strains showed a wide diversity in specific growth rates and oxygen utilization kinetics, and could be divided into four catabolic types of phenol degradation. While some strains degraded phenol mainly via the meta pathway or the ortho pathway, other strains degraded phenol via both these pathways. The ten strains also exhibited high levels of autoaggregation and coaggregation activity. Within the collection of ten strains, 36.7% of all possible strain pairings displayed a measurable degree of coaggregation. Strain PG-08 possessed the strongest autoaggregation activity and showed significant coaggregation (coaggregation indices of 67% to 74%) with PG-02. The three strains PG-01, PG-02 and PG-08 belonging to dominant groups in the granules possessed different competitive characteristics. Microcosm experiments showed the three strains could not coexist at the high phenol concentration of 250 mg/ l, but could coexist at lower phenol concentrations in a spatially heterogeneous environment. This study illustrated that the spatial heterogeneity provided by the aerobic granules led to niche differentiation and increased physiological diversity in the resident microbial community.

Santos *et al.*, (2004) isolated thirty filamentous fungal strains 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.

Rigo *et al.*, (2004) reported that, among 22 species of microorganisms isolated from phenol-containing wastewaters, *Candida parapsilopsis* was found to be capable of growth on a medium with 1 g/L phenol. Kinetic parameters of phenolbiodegradation in a batch reactor were determined by measuring biomass growth rates and phenol concentrationas a function of fermentation time. The Haldane equation described cell growth adequately, withkinetic constants μ_{max} = 0.174/h, K_S = 11.2 mg/L and K_i = 298 mg/L.

Arutchelvan *et al.*, (2005) isolated two bacterial strains capable of utilizing phenol as a sole carbon source from the phenol bearing industrial wastewater. Based on the biochemical test results the organisms were identified as *Pseudomonas cepacia* and *Bacillus brevis*. The organisms were very efficient in phenol degradation, the lag phase increased with increase in phenol concentration. The well-acclimatized cultures of *P. cepacia* and *B. brevis* degraded 2500 and 1750 mg/l of phenol in 144 h, respectively. The organisms degrade phenol even in the presence of toxicants like thiocyanate, sulphide and cyanide. The organisms can be effectively used for treating high strength phenol containing thiocyanate, sulphide and cyanide. The *P. cepacia* degrades phenol with a faster rate than *B. brevis*. *P. cepacia* can be used effectively for treating high strength phenolic wastewater.

Kumar *et al.*, (2005) carried out biodegradation experiments with phenol and catechol using *Pseudomonas putida* MTCC 1194. The bacterial strain used for the degradation experiments were acclimatized with phenol and catechol up to a concentration of 1000 mg/L and 500 mg/L respectively. They observed that the initial phenol concentration of 1000 mg/L and the initial catechol concentration of 500 mg/L were fully degraded in 162 hours and 94 hours,

respectively. Both phenol and catechol exhibited inhibitory behavior and the culture growth kinetics were correlated with Haldane's inhibitory growth kinetic model. They also observed that the bacterial culture died when the initial concentration of phenol and catechol were above 1200 mg/L and 600 mg/L, respectively.

Anli Geng *et al*., (2006) reported the successful isolation and characterization of a new phenol-degrading bacterium, strain EDP3, from activated sludge. Strain EDP3 is a nonmotile, strictly aerobic, Gram-negative, and short-rod or coccobacillary bacterium, which occurs singly, in pairs, or in clusters. 16S rRNA gene sequence analysis revealed that strain EDP3 belonged to the gamma group of *Proteobacteria*, with a 97.0% identity to 16S rRNA gene sequences of *Acinetobacter calcoaceticus*. Strain EDP3 could aerobically grow on a number of aromatic compounds, such as phenol, sodium benzoate, p-hydroxybenzoate, phenylacetate, benzene, ethylbenzene, benzylalcohol, and so on. In particular, it could mineralize up to 1,000 mg l^{-1} phenol at room temperature (25°C). The growth kinetics of strain EDP3 on phenol as a sole carbon and energy source at 25°C can be described using the Haldane equation. It has a maximal specific growth rate (μ_{max}) of 0.28 h^{-1}, a halfsaturation constant (K_S) of 1,167.1 mg l^{-1}, and a substrate inhibition constant (K_i) of 58.5 mg l^{-1}. Values of yield coefficient ($Y_{X/S}$) are between 0.4 and 0.6 mg dry cell (mg phenol)$^{-1}$. Strain EDP3 has high tolerance to the toxicity of phenol (up to 1,000 mg l^{-1}). It therefore could be an excellent candidate for the biotreatment of high-strength phenol-containing industrial wastewaters and for the in situ bioremediation of phenol-contaminated soils.

Arutchelvan *et al.* (2006) isolated and identified a strain of *Bacillus brevis*. Phenol biodegradation in a batch reactor was studied using the pure culture of *B. brevis*. The isolated strain was optimized for various environmental conditions and the biodegradation of phenol was highest at pH 8.0, 5% (v/v) of inoculum size and without any co-substrate. The biokinetic parameters of biodegradation according to Haldane's equation was determined and was found to be μ_{max} = 0.026–0.078 h^{-1}, K_s = 2.2–29.31 mg/l, K_i = 868.0–2434.7 mg/l. These values are specific for this organism and we have compared with literature for pure or mixed cultures degrading phenol.

Karigar *et al*., (2006) studied the ability of *Arthrobacter citreus*, isolated from a hydrocarbon contaminated site, to consume phenol as the sole carbon source. The phenol degradation studies in their work showed that complete degradation of the compound occurred within 24

hours. The organism metabolized phenol with a maximum initial concentration of 22 mM, whereas higher levels were inhibitory.

Lei Wang *et al.*, (2006)isolated a thermophilic *Bacillus* strain NG80-2 growing within the temperature range of 45-73°C (optimum at 65°C) from a deep subterranean oil-reservoir in northern China. The strain was able to utilize crude oil and liquid paraffin as the sole carbon sources for growth, and the growth with crude oil was accompanied by the production of an unknown emulsifying agent. Further examination showed that NG80-2 degraded and utilized only long-chain (C15-C36) n-alkanes, but not short-chain (C8-C14) n-alkanes and those longer than C40. Based on phenotypic and phylogenic analyses, NG80-2 was identified as *Geobacillus thermodenitrificans*. The strain NG80-2 may be potentially used for oily-waste treatment at elevated temperature, a condition which greatly accelerates the biodegradation rate, and for microbial enhancing oil recovery process.

Mailin *et al.*, (2006) isolated four bacteria, two each isolated from wastewater treatment and oil-contaminated soil showed great potential as phenol degraders. RWC-Cr1, the isolate from wastewater was not only demonstrating the highest specific phenol degradation rate at all tested phenol concentrations, but also not affected by the highest phenol concentration employed, namely 1000 mg/l. After a serial transfer of all four isolates into a series of increasing phenol level, all but RWC-Cr1 demonstrated significant improvement on degradation ability. ISC-Tra which was isolated from oil-contaminated soil exhibited greatest improvement (e.g. 75.0% at 600 mg/l phenol) after adaptation in phenol. Thus, the ISC-Tra was the most acclimatizable isolate while in contrary RWC-Cr1 was not significantly influenced by acclimatization. Outcomes of this study offer a useful guideline in evaluating potential phenol degraders from the environment.

Adav*et al.*, (2007) reported that the aerobic granules effectively degrade phenol at high concentrations. This work cultivated aerobic granules that can degrade phenol at a constant rate of 49 mg-phenol/g x VSS/h up to 1,000 mg/L of phenol. Fluorescent staining and confocal laser scanning microscopy (CLSM) tests demonstrated that an active biomass was accumulated at the granule outer layer. A strain with maximum ability to degrade phenol and a high tolerance to phenol toxicity isolated from the granules was identified as *Candida tropicalis* via 18S rRNA sequencing. This strain degrades phenol at a maximum rate of 390

mg-phenol/g x VSS/h at pH 6 and 30 degrees C, whereas inhibitory effects existed at concentrations >1,000 mg/L. The Haldane kinetic model elucidates the growth and phenol biodegradation kinetics of the *C. tropicalis*. The fluorescence in situ hybridization (FISH) and CLSM test suggested that the *Candida* strain was primarily distributed throughout the surface layer of granule; hence, achieving a near constant reaction rate over a wide range of phenol concentration. The mass transfer barrier provided by granule matrix did not determine the reaction rates for the present phenol-degrading granule.

Bai*et al.*, (2007) used a phenol-degrading microorganism, *Alcaligenes faecalis*, to study the substrate interactions during cell growth on phenol and *m*-cresol dual substrates. Both phenol and *m*-cresol could be utilized by the bacteria as the sole carbon and energy sources. When cells grew on the mixture of phenol and *m*-cresol, strong substrate interactions were observed. *m*-Cresol inhibited the degradation of phenol, on the other hand, phenol also inhibited the utilization of *m*-cresol, the overall cell growth rate was the co-action of phenol and *m*-cresol. In addition, the cell growth and substrate degradation kinetics of phenol, *m*-cresol as single and mixed substrates for *A. faecalis* in batch cultures were also investigated over a wide range of initial phenol concentrations (10–1400 mg L^{-1}) and initial *m*-cresol concentrations (5–200 mg L^{-1}). The single-substrate kinetics was described well using the Haldane-type kinetic models, with model constants of $\mu m_1 = 0.15\ h^{-1}$, $KS1 = 2.22$ mg L^{-1} and $Ki1 = 245.37$ mg L^{-1} for cell growth on phenol and $\mu m_2 = 0.0782\ h^{-1}$, $KS_2 = 1.30$ mg L^{-1} and $Ki_2 = 71.77$ mgL^{-1}, $K'_{i2} = 5480\ (mg\ L^{-1})^2$ for cell growth on *m*-cresol. Proposed cell growth kinetic model was used to characterize the substrates interactions in the dual substrates system, the obtained parameters representing interactions between phenol and *m*-cresol were, $K = 1.8 \times 10^{-6}$, $M = 5.5 \times 10^{-5}$, $Q = 6.7 \times 10^{-4}$. The results received in the experiments demonstrated that these models adequately described the dynamic behaviors of phenol and *m*-cresol as single and mixed substrates by the strain of *A. faecalis.*

Cai*et al.*, (2007) reported that the *Fusarium sp*. HJ01 can grow using phenol as only carbon resource and has strong ability of phenol degradation. The effect of pH, temperature and sucrose addition on biodegradative capacity of *Fusarium sp*. HJ01 was examined. The main metabolism pathways and mechanism of phenol degradation by HJ01 strain is described. This strain exhibited both cathecol 1,2-dioxygenase (C12) and cathecol 2,3-dioxygenase (C23) in free cell extracts obtained from cells grown exclusively on phenol or with sucrose added,

suggesting that the intermediate cathecol can be oxidized in the catabolic pathway of ortho and meta fission. Mineral salts added in culture have an inhibition on both C12 and C23. These two enzymes can act and retain its catalytic ability over wide ranges of temperature and pH. C12 activity was optimal at pH 6.8 and 40 degrees C, with significant activity observed in the range from pH 3 to pH 8.8, and in the temperature range from 30 to 50 degrees C. In comparison with C12, the activity of C23 was slightly more sensitive to pH, C23 had a higher activity in alkalescence condition from pH 7.4 to pH 10.6 and was more stable at higher temperatures from 30 to 75 degrees C.

Jiang *et al.*, (2007) isolated a strain of *Alcaligenes faecalis* from activated sludge collected from a municipal gasworks. The phenol biodegradation tests showed that the phenol-degrading potential of *A. faecalis* related greatly to the different physiological phases of inoculum. The maximum phenol degradation occurred at the late phase of the exponential growth stages, where 1600 mg L^{-1} phenol was completely degraded within 76 hours. *A.faecalis* secreted and accumulated a vast quantity of phenol hydroxylase in this physiological phase, which ensured that the cells could quickly utilize phenol as a sole carbon and energy source. In addition, the kinetic behavior of *A. faecalis* in batch cultures was also investigated over a wide range of initial phenol concentrations (0–1600 mg L^{-1}) by using Haldane model. It was clear that the Haldane kinetic model adequately described the dynamic behavior of the phenol biodegradation by the strain of *A. faecalis*.

Agarry *et al.*, (2008) studied the bioremediation potential of an indigenous *Pseudomonas fluorescence* in batch culture using synthetic phenol in water in the concentration range of (100 –500) mg/L as a model limiting substrate. The effect of initial phenol concentration on the degradation process was investigated. Phenol was completely degraded at different cultivation times for the different initial phenol concentrations. Increasing the initial phenol concentration from 100 mg/L to 500 mg/L increased the lag phase from 0 to 66 hours and correspondingly prolonged the degradation process from 84 hours to 354 hours. There was decrease in biodegradation rate as initial phenol concentration increased. Fitting data into Monod kinetic model showed the inhibition effect of phenol. The kinetic parameters have been estimated up to initial phenol concentration of 500 mg/ L. The rs_{max} decreased and K_s increased with higher concentration of phenol. The biokinetic constants estimated using Haldane model showed good potential of the *Pseudomonasfluorescence* and the possibility of using it in bioremediation of phenol waste effluents.

Indu Nair *et al.*, (2008) carried out their experiments on biodegradation of phenol. It was found that large numbers of microbes co-exist in almost all natural environments, particularly in soils. Many synthetic and natural organic chemicals are readily biodegradable in natural environment. They observed that biodegradation of materials involve allowing adsorption or physical access to the substrate, initial proximity, secretion of extra cellular enzymes to degrade the substrates or uptake via transport systems followed by intracellular metabolism. They observed that the efficiency of biodegradation of organic compounds is influenced by the type of the nature of the organism, the organic pollutant, the enzyme involved, the nature of the influencing factors and the mechanism of degradation. Phenolic compounds are toxic at relatively low concentration and also they are hazardous pollutants. Accumulation of phenol creates toxicity both for plants and animals. Since phenol is a toxic substance and also it cause pollution, therefore it must be removed from the environment. It was found that *K. oxytoca* degraded phenol at elevated concentration where 75% of initial phenol concentration at 100 ppm was degraded within 72 h (Shawabkeh et al., 2007). Phenol was degraded by *Actinobacillus* species (Khleifat and Khaled, 2007). They found that optimal conditions for maximum degradation of phenol are at pH of 7, the incubation temperature of 35 to 37°C, and the agitation rate of 150 rpm.

Kotresha and Vidyasagar (2008) isolated a novel indigenous *Pseudomonas aeruginosa* strain (MTCC 4996) from a pulp industrial effluent-contaminated site that was capable of degrading phenol up to a concentration of 1,300 mg L^{-1} within 156 h. Complete degradation was observed at pH values ranging from 6.0 to 10.0 and temperatures from 15 to 45°C, with an optimum pH of 7.0 and optimum temperature of 37°C. At an optimum shaking speed of 100–125 rpm, 100% degradation was observed in 66 h, as compared to 84 h under static conditions. Glucose and peptone at lower concentrations enhanced phenol degradation. The rate of phenol degradation was most sensitive to added Hg. Low concentrations of Fe, Cu, Pb, Zn, and Mn stimulated and enhanced the rate of degradation.

Sidra ilyas *et al.*, (2008)investigated the ability of *Bacillus megaterium*and *Micrococcus luteus*to degrade phenol into non-toxic form. Both*Bacillus megaterium*and *Micrococcus luteus*could tolerate phenol up to 300µg/ml. *Bacillus megaterium*and *Micrococcus luteus*both showed optimum growthat pH 7 while the maximum growth of both bacteria was observed at 37ºC. Bothbacterial isolates, Bacillus *megaterium* and *Micrococcus luteus*, showed high 2,3-

dioxygenase activity of 50.5% and 52.3%, respectively. *Bacillus megaterium* and*Micrococcus luteus*could degrade 64% and 70% of phenol (100µg/ml) from themedium after 12 h. The bacterial isolates can be exploited for bioremediation ofphenol and phenol derivates containing wastes, since they seem to have thepotential to degrade enzymatically the toxic phenol into non-toxic product form.

Rubilar *et al.*, (2008) analyzed the degradation of chlorophenols by white rot fungi, which are a group of organisms very suitable for the removal of chlorinated phenolic compounds. They are robust organisms that are tolerant to the presence of high concentrations of various pollutants, even with a low bioavailability and this ability is mainly due to their very powerful extracellular oxidative enzymatic system.

Wenjing *et al.*, (2008) isolated 46 phenol / benzoate degrading and iron reducing bacteria from long term irrigated tropical paddy soils by enrichment procedures. Pure cultures and some prepared mixed cultures were examined for ferric oxide reduction and phenol/benzoate degradation. All the isolates were iron reducers, but only 56.5% could couple iron reduction to phenol and/or benzoate degradation, as evidenced by depletion of phenol and benzoate after one week incubation. Analysis of degradative capability using Biolog MT plates revealed that most of them could degrade other aromatic compounds such as ferulic acid, vanillic acid, and hydroxybenzoate. Mixed cultures and soil samples displayed greater capacity for aromatic degradation and iron reduction than pure bacterial isolates. Bacteria capable of coupling these reactions may be major contributors to the microbial cycling of large molecule carbon substrates and are thus promising tools for bioremediation and elimination of organic pollutants in Fe mediated anaerobic simulators.

Cordova-Rosa *et al.*, (2009) reported about the time-course performance of a phenol degrading indigenous bacterial consortium, and of *Acinetobacter calcoaceticus* var. *anitratus*, isolated from an industrial coal wastewater treatment plant. The bacterial consortium was able to survive in the presence of phenol concentrations as high as 1200mgL^{-1} and the consortium was faster in degrading phenol than a pure culture of the *A.calcoaceticus* strain. A high phenol biodegradation (above 95%) by the mixed culture in a bioreactor was obtained in both continuous and batch systems, but when test was carried out in coke gasification wastewater, no biodegradation was observed after 10 days at pH 9–11 for both pure strain or the isolated consortium.

Kilic *et al.*, (2007; 2009) reported the isolation and characterization of the bacterium *Ochrobactrum* sp. It was metabolized phenol through catechol, followed by ortho- or meta-pathways (El-Sayed *et al.*, 2003). There is no report investigating phenol degradation capacity and the conditions affecting phenol degradation by *Ochrobactrum* sp. Furthermore, some other phenolic compounds like 4-nitrocatechol (Zhong *et al.*, 2007), p-nitrophenol (Qiu *et al.*, 2007), and 2,4,6- tribromophenol (Yamada *et al.*, 2008) were metabolized by *Ochrobactrum* species.

Shourian *et al.*, (2009) isolated a potent phenol-degrading bacterium, assigned *Pseudomonas* sp. SA01 from pharmaceutical disposal wastewaters plant. According to biochemical characteristics and 16S rRNA sequence analysis, the isolate was identified as *Pseudomonas* sp. The isolated strain started to degrade 0.7 g/l of phenol after an initial very short lag phase, and phenol decomposition was then rapidly completed within 30 hours. *Pseudomonas* sp.SA01 was able to degrade phenol in concentrations up to 1 g/l. Higher phenol concentrations (>1 g/l) had a significant inhibitory effect on bacterial growth. The optimum degradation pH value was found to be 6.5. Addition of mannitol and casein as auxiliary carbon and nitrogen sources enhanced the rate of phenol removal to as low as 20 hours. Based on the absorption spectra of catechol bioconversion of phenol-grown cells, it was concluded that the SA01 strain metabolizes the phenol via a meta-cleavage pathway.

Aresta *et al.*, (2010) isolated 28 bacterial strains from greenwaters-polluted-soil and investigated for their ability to grow in presence of phenols added to Mineral Basal Medium (MBM) in aerobic conditions. In particular, three of them were found to be able to use as sole carbon source phenol, cathecol, caffeic acid and ferulic acid with efficiency ranging from 76% (phenol in 5 days, millimolar concentration from 3.7 $x10^{-2}$ to 9 $x10^{-3}$) to 95% (ferulic acid in 2 days millimolar concentration from 6.8 $x10^{-1}$ to 3 $x10^{-2}$). For these strains the taxonomic position was studied by amplification and sequencing of 16S rRNA genes. The isolated strains were classified belonging to *Arthrobacter sulfureus*, *Pseudomonas synxantha* and *Pseudomonas oryzihabitans.* Noteworthy, for the first time such *Pseudomonas* strains have been shown to be able to use polyphenols as the only carbon source in vitro. This kind of study were not done on *Ps. Synxantha*, while it was recently shown the ability of *P. oryzihabitans* to degrade catechol. These findings may open to new biotechnological applications for the degradation of polyphenols.

Banerjee*et al.,* (2010) studied the microbial degradation of phenol by pure cultures *Bacilluscereus* MTCC 9817 strain AKG1 and *B. cereus* MTCC 9818 strain AKG2 in batch mode for several initial concentrations of phenol in the range of 100-2000 mg/L with an interval of 100mg/L. Degradation pathways are investigated at initial phenol concentrations of 100, 500, 1000, 1500, and 2000 mg/L. The bacteria are able to degrade phenol of concentration as high as 2000 mg/L. The maximum degradation rate is obtained at an initial phenol concentration of about 800 mg/L for the strain AKG1 and about 200mg/L for the strain AKG2. Both the strains degrade phenol via meta-cleavage pathway through formation of 2-hydroxymuconic semialdehyde (2-HMSA) as an intermediate product. Modeling of the biodegradation of phenol indicates that the Haldane inhibitory model predicts the experimental data fairly well for both the strains.

Bhavna *et al.,* (2010)isolated the aerobic bacteria from soil contaminated with industrial xenobiotic compounds using enrichment technique containing phenol as sole source of carbon and energy in pure culture and selected for their ability to degrade phenol. The soil bacterium was identified as *Streptococcus epidermis* coded as (OCS-B). The selected microbial strain was able to degrade phenol up to 200mg/l which was also confirmed by HPLC analysis and so can be effectively used for bioremediation of phenol contaminated sites. Degradation intermediate compounds were also determined. Outcome of this study offer a useful guideline in evaluating potential phenol degraders from the environment.

Chakraborty *et al.,* (2010) studied the isolation of phenol by native bacteria strains from coke oven processing wastewater to assess the biodegradation. The strains were designated ESDSPB1, ESDSPB2 and ESDSPB3 and examined for colony morphology Gram stain characters and biochemical tests. Phenol degrading performance of all the strains was evaluated initially. One of the strains namely ESDSPB2 was found to be highly effective for the removal of phenol, which was used as sole carbon and energy source. From an initial concentration of 200 mg l^{-1} it degraded to 79.84 ± 1.23 mg l^{-1}. In turn the effect of temperature (20 to 45^0C), pH (5 – 10) and glucose concentration (0, 0.25 and 0.5%) on the rate of phenol degradation by that particular strain was investigated. Observations revealed that the rate of phenol biodegradation was significantly affected by pH, temperature of incubation and glucose concentration. The optimal conditions for phenol removal were found

to be pH of 7 (84.63% removal), temperature, 30^0C (76.69% removal) and 0.25% supplemented glucose level (97.88% removal).

Jame *et al.*, (2010) isolated four different *Pseudomonas* species viz FA, SA, TK and KA. All these isolates could completely degrade phenol up to 600ppm. Isolate *Pseudomonas* FA degraded 800ppm phenol completely in 72 h, but the isolates *Pseudomonas* SA, TK and KA degraded only 39.33, 43.83 and 33.16% of 800ppm phenol respectively in 96 h. From mixed cultures of *Pseudomonas putida* A (a) and *Pseudomonas* sp. SA, it was found that *P. putida* A (a) degraded 600ppm phenol in 24 hour and *Pseudomonas* sp. SA degraded the same concentration in 72 h when they were cultured individually. The mix culture of this two *Pseudomonas* sp. degraded the same concentration in 20 h.

Farshid Kafilzadeh *et al.*, (2010)carried out their study on isolation and identification of phenol degrading bacteria from Lake Parishan and to assay their kinetic growth. Sixty samples of water and sedimentation of different area of Lake Parishan were collected. In order to isolate phenol degrading bacteria, samples were cultured on salt base phenol broth media. For screening of degrading bacteria, bromothymole blue indicator was added to media, which formed green color in it. Finally, the ability of bacteria to degrade different concentration of phenol was measured using culturing bacteria in different concentration of phenol from 0.2 to 0.9 g/l. Cultivated bacteria on the salt base phenol broth containing indicator changed the color of the media from green to yellow by using the phenol and decreasing the pH. These bacteria were, chiefly, gram negative and they belong to *Pseudomonaceae* and *Acinetobacteraceae* Family. *Pseudomonas* spp. are the most important phenol degrading bacteria in Lake Parishan which showed vast diversity in different parts of this lake. Species of *Acinetobacter* and other species such as *Kelibsiella, Citrobacter* and *Shigella* were found as well. Most of the isolated bacteria showed a good ability of degradation of phenol, where *Pseudomonas* and *Acinetobacter* showed 0.8 - 0.9 g/l, and *Kelibsiella*, *Citrobacter* and *Shigella* showed 0.6 - 0.7 g/l and the rest showed 0.2 - 0.3 g/l of phenol degradation. Findings show that the Lake Parishan has a lot of high ability phenol degrading bacteria. The most important species belong to *Pseudomonas* and *Acinetobacter*.

Razika *et al.*, (2010)conducted studies on bacterium *Pseudomonas aeruginosa* to eliminate phenol and the benzoic acid and reported that the microbe is able to degrade phenol as well as benzoic acid. However, it was noted that *Pseudomonas aeruginosa* shows better results in

phenol than benzoic acid. However, *Pseudomonas aeroginosa* could not degrade very high concentrations of phenol and benzoic acid completely (> 80 mg/l).

Chandana Lakshmi *et al.*, (2011) selected *Pseudomonas aeruginosa* (NCIM 2074) which can utilize phenol as a sole source of carbon and energy was selected for the degradation of phenol. Experiments were made as a function of carbon source (glucose), inorganic nitrogen (ammonium chloride) and metal ion concentration (zinc ion). In this work, a 23- full factorial Central Composite Design was employed combining with Response Surface Methodology (RSM) to optimize the process parameters for the degradation of phenol by *P.aeruginosa* (NCIM 2074). It was shown that a second order polynomial regression model could properly interpret the experimental data with an R2 value of 0.9669 and an F-value of 32.52295 based on which the maximum degradation of phenol was estimated up to 80.45% within the range examined.

Hong-xia (2011) isolated 15 different bacterial strains from marine sources on the beef extract peptone agar plates with 1500mgL^{-1} phenol. Among them, the strain SM5 could tolerate 4500 mg/L phenol on solid beef extract peptone plates and its phenol biodegradation rate was 96.4% in basal salt (BS) medium under the optimum conditions when the concentration of phenol was 1000 mg/L. These conditions were; initial pH 7.0, 37°C, 3 days, 20 ml medium/50 ml flask and inoculums biomass 12.5% (v/v). Rate of phenol biodegradation of the strain was up to 92.0% under the optimum conditions even when the phenol concentration was increased to 2500 mgL^{-1}. *Staphylococcus aureus* was isolated from effluent sample by enrichment of the effluent. This isolated bacterium was tested for its potential of phenol remove from effluent by added 1000ppm of phenol to Bushnell Haas (BH) medium as a sole source of carbon and nitrogen.

Butani Naresh *et al.*, (2012)noticed the native bacterial strain isolated from phenol contaminated site of Amla Khadi, Ankleshwar and used to study biodegradation of phenol in shake flask culture. Various physicochemical parameters are optimized for the maximum biodegradation of phenol, viz., pH, temperature, initial concentration of phenol, additional carbon sources and additional nitrogen sources. Complete phenol biodegradation was achieved after 4 days in 1000 ppm solution. The isolated Gram positive bacterium can be exploited as a candidate of choice for the bioremediation of phenolic effluent.

Han Ba Bui1,(2012)noticed and selected the aerobic bacteria in soil contaminated with dioxin (taken from Da Nang airport's area in Vietnam) were isolated and selected for their ability to degrade phenol using enrichment technique containing phenol as sole source of carbon and energy (100 mg/L phenol in a mineral salt medium). Four strains (designated D1.1, D1.3, D1.4, and D1.6) were obtained and characterized. The results showed that these bacteria were highly effective for the removal of phenol. After 120 hours of culture, strain D1.4 degraded 54.84% and 44.19% phenol from the initial concentrations of 100 mg/L and 1000 mg/L, respectively; strain D1.6 degraded 66.45% of phenol from the initial concentration of 1500 mg/L. The combination of those bacteria in the same medium had a positive effect on the phenol degradation activity. The outcome of the study can contribute new useful resources for treatments of wastewater and soils contaminated with phenolic wastes.

Tambekar *et al.*, (2012) isolated a phenol degrader from Lonar Lake situated in Buldhana district of Maharashtra state. From 4 sediments and water samples of Lonar Lake, a bacterium *Pseudomonas stutzeri* was isolated and identified based on morphological, cultural, biochemical properties and 16S rRNA gene sequencing. The experiment showed that the isolate removes almost 87% phenol in the peptone water-phenol medium at laboratory level. Thus, the alkaliphilic bacterium, *Pseudomonas stutzeri*, ensuring an acceptable Lonar lake bacterium, which could therefore be commercially exploit for bioremediation of phenol, a major toxic pollutant in industrial waste effluents.

Dupadahalli Kotresha *et al.*, (2013)conducted experiments on isolation of phenol degrading bacterial strains from industrial effluents in order to know the suitable phenol degrading bacterial strain, 65 bacterial strains were isolated from different places of industrial effluents from textile, pulp mills, paper industry, distillery, petrochemical, dye industries and sewage water and studied their potential in degradation of phenol. Among the 65 isolates, 23 isolates degraded 500 mg/l phenol, 25 isolates degraded 500 to 1000 mg/l phenol, 15 isolates degraded 1000 mg/l to 1200 mg/l phenol. Isolates PVK-12 and PVK-26 were found to be more efficient as compared to other isolates, both these isolates utilizes phenol as a sole source of carbon and energy and capable of phenol degradation up to 1300 mg/l and 1400 mg/l respectively.

Krastanov*et al.*, (2013) were carried out their study on Microbial degradation of phenol and phenolic compounds. A number of microbial species possess enzyme systems that are applicable for the decomposition of various aliphatic and aromatic toxic compounds. Intensive efforts to screen species with high-degradation activity are needed to study their capabilities of degrading phenol and phenolic derivatives. Most of the current research has been directed at the isolation and study of microbial species of potential ecological significance. In this review, some of the best achievements in degrading phenolic compounds by bacteria and yeasts are presented, which draws attention to the high efficiency of strains of *Pseudomonas, Candida tropicalis, Trichosporon cutaneum*, etc. The unique ability of fungi to maintain their degradation potential under conditions unfavorable for other microorganisms is outstanding. Mathematical models of the microbial biodegradation dynamics of single and mixed aromatic compounds, which direct to the benefit of the processes studied in optimization of modern environmental biotechnology are also presented.

Shwetha *et al.*, (2013) optimized bioremediation process to enhance the phenol removal from aqueous solution by a newly isolated and biochemically identified bacteria *Pseudomonas putida* from an industry effluent. This bacterium was capable of utilizing phenol as a sole source of carbon and was very efficient for phenol degradation at optimized culture condition. 1000 mg.l-1 of phenol was completely degraded at 60th hour of contact time. The optimum pH and temperature for highest phenol degradation was 7 and 35^0C respectively. Media was supplemented with different concentrations of Iron (Fe) and Selenium (Se) for the growth of *Pseudomonasputida* and phenol degradation. Maximum phenol degradation of 79.7 % and 80.3% was observed at 0.5mg/l Fe and 0.5mg/l Se respectively. From the results, we conclude that the phenol biodegradation and cultural parameters are interdependent by statistical analysis.

Yaacob Nor Suhaila *et al.*, (2013) studied Response surface methodology (RSM) to optimize medium composition and culture condition for enhancement of growth of *Rhodococcus UKMP-5M* and phenol degradation rate in shake flask cultures. Phenol and (NH4)2SO4 concentrations as well as temperature were the most significant factors that influenced growth and phenol degradation. Central composite design (CCD) was used for optimization of these parameters with growth, and degradation rates were used as the responses. Cultivation with 0.5 g/L phenol and 0.3 g/L $(NH_4)_2SO_4$ and incubation at 36 °C

greatly enhanced growth of *Rhodococcus UKMP-5M*, where the final cell concentration increased from 0.117 g/L to 0.376 g/L. On the other hand, the degradation rate was greatly increased in cultivation with 0.7 g/L phenol and 0.4 g/L $(NH_4)_2SO_4$ and incubation at 37 °C. In this cultivation, the time taken to degrade 1 g/L phenol in the culture was reduced from 48 h to 27 h. The model for both responses was found significant and the predicted values were found to be in a good agreement with experimental values and subsequently validated.Increases in phenol degradation rate during *Rhodococcus UKMP-5M* cultivation corresponded well with increasing phenol hydroxylase activity.

Kotresha and vidhyasagar (2014) isolated a novel strain of *Pseudomonas aeuriginosa* MTCC 4997 from effluents collected from petrochemical industries. The isolated strain was optimized for various operational and environmental conditions in batch culture. This strain was utilizes phenol as a sole source of carbon and energy, capable of degrading phenol up to 1400 mg l-1 concentration within 144 h. Complete degradation was observed at wide temperature from 15^0C to 45^0C with an optimum of 37^0C and pH 6.0 to 10.5 with an optimum of 7.0 to 7.5. Metals such as Fe, Cd, Cu, Pb, Zn, Mn and Ba at lower concentrations were found stimulated and enhanced the rate of phenol degradation. Therefore, the phenol degradability of the strain can be maintained and used at large scale treatment.

Maulin P Shah (2014) investigated the assessment of phenol biodegradation by bioaugmentation of *Pseudomonas spp. ETL-2412.* The strain was isolated and designated as *Pseudomonas spp. ETL 2412* after examined for colony morphology, gram stain characteristics and various biochemical tests. *Pseudomonas spp. ETL 2412* was found to be highly effectual for the removal of phenol which was used as sole carbon and energy source. From an initial concentration of 200 mg l-1 it degraded to 76.43 ± 1.23 mg l-1. In turn the effect of temperature (25 to 50°C), pH (5.5 – 10.5) and glucose concentration (0, 0.25 and 0.5%) on the rate of phenol degradation was investigated. Observations revealed that the rate of phenol biodegradation was affected by pH, temperature and glucose concentration. The optimal conditions for phenol removal were found at pH 7.5 (82.63%), temperature 30°C (78.69%) and 0.25% supplemented glucose level (98.28%). It can be concluded that this strain has remarkable potential for application in bioremediation and wastewater treatment, especially in detoxification of phenolic waste. The significance & impact of the study is the utilization of native bacterial strains isolated from the waste water itself having potential for environmental bioremediation in the activated sludge process of a FETP Plant.

Negar Amini Boroujeni *et al.*, (2014) studied the isolation of the phenol degrading bacteria from marine environmental samples (soil and water) from the Persian Gulf. After three passages, the bacterial growth was measured that four bacteria (F6, F10, F13, F16) has the highest rate of growth. Also, these bacteria were able to remove phenol that was measured by absorbance at 272 nm. The hydrophobicity and emulsification activity was measured in all four bacteria. Finally, after a series of biochemical tests, molecular analysis for strong bacteria in degrading phenol, 16S rRNA gene region amplified with primers specific part of the gene was performed. The sequence result of the gene bank and the highest homology (greater than 98%) were identified as species of bacteria. Genus of isolated bacteria was belonging to *Nitratireductor aquimarinus, Nitratireductor aquimarius,* Marine bacterium, *Pseudomonasstutzeri.*

Prabhu and Narendrababu (2015) studied optimization of process parameters for removal of phenol by nano zero valent iron impregnated cashew nut shield by adopting statistical tool Response Surface Methodology-Box Behnken Design (RSM-BB) to explore the effect of variables on the removal of phenol. In RSM-BB method, high and low values were assigned for the five variables viz. initial pH, NZVI-CNS dosage, initial concentration, contact time and temperature. The preparation was carried out by simple liquid-phase reduction method, namely borohydride reduction method. They reported that this method showed the significant effect of pH (A), Dose (B), initial concentration (C), time (D), and temperature (E) on phenol removal from aqueous solution. The results of ANOVA and regression of the second order model showed that the linear effects of Dose (B) and Temperature and cross products effects of temperature and pH were more significant. All the critical variables having the greatest effect on the removal of phenol from Nano zero valent iron impregnated cashew nut shell. He concluded that nano zero valent iron impregnated cashew nut shell successfully employed to remove phenol from aqueous solution. The factors optimized in the present work would helpful in phenol removal from aqueous solution

Sangram *et al*., (2015) reported that the parameters such as initial phenol concentration, pH, temperature, inoculum size, and concentration of various medium components largely affect the phenol degradation ability of microbes; hence, these parameters must be optimized in order to achieve maximum phenol degradation. They have studied the optimization of phenol degradation by *Bacillus pumilus* OS1, isolated from soil of crude oil spillage site.

Experimental design methodology has been adopted for the optimization study. The Plackett–Burman design has determined five significant factors [pH, temperature, phenol concentration, inoculum size, and $(NH_4)_2SO_4$ concentration out of the nine variables, important for phenol degradation. Response surface analysis using central composite design has been used to study mutual interactions between these variables and to find their optimum levels. The predicted result shows that maximum phenol degradation (99.99%) could be achieved at pH 7.07, temperature 29.3 ºC, phenol 227.4mg/l, inoculum size 6.3% (v/v), $(NH_4)_2SO_4$ 392.1 mg/l. The correlation coefficient (R^2= 0.9679) indicates an excellent agreement between the experimental values and predicted ones. A fairly good agreement between the model predicted value and the one obtained from subsequent experimentation at the optimized conditions confirms the validity of the model.

Qihui Gu et al., (2016) reported that Phenol is a ubiquitous organic contaminant in drinking water. Biodegradation plays an important role in the elimination of phenol pollution in the environment, but the information about phenol removal by drinking water biofilters is still lacking. Herein, we study an acclimated bacterial community that can degrade over 80% of 300 mg/L phenol within 3 days. PCR detection of genotypes involved in bacterial phenol degradation revealed that the degradation pathways contained the initial oxidative attack by phenol hydroxylase, and subsequent ring fission by catechol 1,2-dioxygenase. Based on the PCR denatured gradient gel electrophoresis (PCR-DGGE) profiles of bacteria from biological activated carbon (BAC), the predominant bacteria in drinking water biofilters including *Delftia* sp., *Achromobacter* sp., and *Agrobacterium* sp., which together comprised up to 50% of the total microorganisms. In addition, a shift in bacterial community structure was observed during phenol biodegradation. Furthermore, the most effective phenol-degrading strain DW-1 that correspond to the main band in denaturing gradient gel electrophoresis (DGGE) profile was isolated and identified as *Acinetobacter* sp., according to phylogenetic analyses of the 16S ribosomal ribonucleic acid (rRNA) gene sequences. The strain DW-1 also produced the most important enzyme, phenol hydroxylase, and it also exhibited a good ability to degrade phenol when immobilized on granular active carbon (GAC). This study indicates that the enrichment culture has great potential application for treatment of phenol-polluted drinking water sources, and the indigenous phenol-degrading microorganism could recover from drinking water biofilters as an efficient resource for phenol removal. Therefore, the aim of this study is to draw attention to recover native phenol-

degrading bacteria from drinking water biofilters, and use these native microorganisms as phenolic water remediation in drinking water sources.

Satya Sundar Mohanty et al., (2017) studied that a pure culture of bacterium (Pseudomonas sp. Strain NBM11) was isolated from the soil sample from a site contaminated with medical wastes and wastewater. The isolated strain can degrade up to 1000 mg/L of phenol completely. It was observed that temperature, pH and initial concentration of phenol play key roles in determining the rate of phenol degradation. The isolated strain exhibited the maximal degradation of the substrate within a range of pH 6.8 to 7.2 and an incubation temperature between 30 ºC and 32 ºC. It was found that by increasing the concentration of phenol, the lag phase gets extended due to the inhibitory nature of phenol. The kinetic parameters such as μmax (maximum specific growth rate), Ks (half-saturation coefficient) and Ki (substrate inhibition constant) were estimated as 0.184 1/h, 7.79 mg/L and 319.24 mg/L, respectively, by fitting the growth kinetics data to the Haldane model of substrate inhibition. The bacterial strain was immobilized in alginate beads and its phenol degradation efficiency was observed to increase many fold. The immobilized cells were found to be used efficiently for seven cycles consecutively without any decrease in their efficiency.

Mengyang Tian et al., (2017) stated that The aerobic degradation of aromatic compounds by bacteria is performed by dioxygenases. To show some characteristic patterns of the dioxygenase genotype and its degradation specificities, twenty-nine gram-negative bacterial cultures were obtained from sediment contaminated with phenolic compounds in Wuhan, China. The isolates were phylogenetically diverse and belonged to 10 genera. All 29 gram-negative bacteria were able to utilize phenol, m-dihydroxybenzene and 2-hydroxybenzoic acid as the sole carbon sources, and members of the three primary genera *Pseudomonas*, *Acinetobacter* and *Alcaligenes* were able to grow in the presence of multiple monoaromatic compounds. PCR and DNA sequence analysis were used to detect dioxygenase genes coding for catechol 1,2-dioxygenase, catechol 2,3-dioxygenase and protocatechuate 3,4-dioxygenase. The results showed that there are 4 genotypes; most strains are either PNP (catechol 1,2-dioxygenase gene is positive, catechol 2,3-dioxygenase gene is negative, protocatechuate 3,4-dioxygenase gene is positive) or PNN (catechol 1,2-dioxygenase gene is positive, catechol 2,3-dioxygenase gene is negative, protocatechuate 3,4-dioxygenase gene is negative). The strains with two dioxygenase genes can usually grow on many more aromatic compounds than strains with one dioxygenase gene. Degradation experiments using a mixed culture representing four bacterial genotypes resulted in the rapid

degradation of phenol. Determinations of substrate utilization and phenol degradation revealed their affiliations through dioxygenase genotype data.

4. Materials and Methods

4.1. Sample Collection:

The effluent samples were collected fromTextile industry,Visakhapatnam, Andhra Pradesh.

4.1.1. Sample Preparation:

The samples were collected in sterile bottles Stored it in refrigerator at 4°C. All the samples were used within 7 days from the day of collection for bacteriological analysis. The water sample Bottles were mechanically shaken prior to use and Kept for 10 mins to allow heavy particles to settle down.The approximate volume of upper layer of water was taken for bacteriologicalanalysis.

4.2. Fourier Transform Infrared Spectroscopy (FTIR) analysis:

FT-IR is used to determine the different functional group such as alcohol, alkane, alkynes, alkenes and other such groups present in the substance which here is Phenol. Interpreting infrared (IR) spectra is of immense help to structure determination. Not only will it tell you what functional groups and structural elements are there, it will also clarify which ones are present, and also concentration of bands by using values of transmittance.

The samples were prepared with the dimension of 10 x 4 x 4. Samples were removed from water and air dried, and scraped using sharp, clean sterile wax knife to obtain powder of the polymerized samples.

4.2.1. Working principle:

- FTIR transmits infrared light through a sample and varies the wavelength of the light.
- The light is absorbed at different wavelengths gives spectra.
- The obtained spectra is compared with the computerized database contains many known spectra for thousand of organic materials by taking frequency on X- axis and absorbance on Y- axis.

4.3. Steam Distillation: Steam distillationis a special type of distillation (a separation process) for temperature sensitive materials like natural aromaticcompounds. It once was a popular laboratory method for purification of organic compounds, but has become obsolete by vacuum distillation. It is an efficient way to remove organic compounds or Volatile Organic Compounds (VOC's) from a water-based process stream. Generally speaking a pound of steam will vaporize 4-5 pounds of organics because steam has a latency of vaporization of 1000's BTUs/lb vs. 200 BTUs for organics.

4.3.1. Principle:

When a mixture of two practically immiscible liquids is heated while being agitated to expose the surface of each liquid to the vapor phase, each constituent independently exerts its own vapor pressure as a function of temperature as if the other constituent were not present. Consequently, the vapor pressure of the whole system increases. Boiling begins when the sum of the vapour pressures of the two immiscible liquids just exceeds the atmospheric pressure (approximately 101 kPa at sea level).

4.3.2. Steam distillation setup:

The steam distillation set up that contains the Boiling Flask (1L),Biomass Flask (2L),and Distillation Arm,Glass Stopper for Distillation Arm, Condenser andErlenmeyer flask. The hot water in the boiling flask that will generate steam and thus will provide an internal source of steam. Add to the boiling flask at least three times as much as water as sample. Do not fill the flask much more than half full. Periodically, add more hot water as needed, when the water boils and turns to steam, it also leaves the flask, carrying the product. Often times, while a distillation is in progress, steam may condense on the sides of the biomass flask and the distillation arm before it makes it's way into the condenser. This is simply a property of a distillation system that contains a large surface area of glass. Hot steam tends to condense back into water as soon as it can. To minimize the amount of steam that condenses too soon and thus drips back into the boiling flask, insulation like cloth or foam may be wrapped around the biomass flask and distillation arm. Additionally, make sure that the plant material in the biomass flask is not packed too tightly. In any case, some steam will inevitably condense and drip back into the boiling flask during operation. It is typical for some of the boiling water to remain (or drip back down) in the boiling flask for the duration of the distillation. This water may also take on a yellow to

brown color as water soluble components of the material are released from the plant during distillation. Monitoring the progress of the distillation is a skill that requires practice, patience, and research into the specifics of the plant material being used. Many people prefer to end their distillation when the hydrosol water dripping from the end of the condenser loses the pleasant aromas prominent at the beginning stages of the process. Again, it is not unusual that some water will remain in the boiling flask when it is time to end a successful distillation. The distillation set up was shown in the figure 4.1.

Fig4.1. Steam Distillation Setup

1. Boiling Flask (1L),2. Biomass Flask (2L) 3. Distillation Arm, 4. Condenser
5. Glass Stopper 6. Erlenmeyer flask.

4.4. Isolation and Screening of Phenol degrading microorganisms:

The process consists of the following steps:

Media Preparation → Sterilization → Culturing → Inoculation →

4.4.1. Media preparation: MSAM and MSM composition was added in 500 ml of distilled water in a conical flask.

- **Media Used For Isolation:**

The following media was used for isolation of potential. The composition of MSAM medium was shown in Table 4.1.

MSAM Media (Mineral salt Agar Medium)

Table 4.1. Composition of Mineral salt Agar medium

Chemical Composition	g/L
Sodium nitrate	2.0
Magnesium Sulfate	0.5
Potassium Chloride	0.5
Ferric sulfate	0.01
Pottasium Dihydrogen Phosphate	1.5
Dipotassium Hydrogen Phosphate	1.7
Agar	15
Yeast Extract	2.0
Sodium Sulfate	3.0
Sodium Chloride	0.5
Calcium Chloride	0.5
Ammonium Chloride	0.5
Nutrient Agar	0.24
P^H	7.2

Maintenance Medium:

The composition of maintenance medium was shown in Table 4.2.

MSM Medium(Mineral salt media)

Table 4.2. Composition of mineral salt medium

Chemical Composition	g/L
KH_2PO_4	1.5
K_2HPO_4	0.5
NH_4Cl	0.5
$CaCl_2$	0.02
$MgSO_4.\ 7H_2O$	0.2
NaCl	0.5
Na_2SO_4	3.0
Yeast Extract	2.0
Nutrient Agar	2.0

4.4.2. Heat Sterilization: It is the process, in which foreign contamination is removed by means of heat, in lab Auto-clave was used for heat sterilization.

- After the preparation of MSAM, it was kept in auto-clave. A pressure of 15-20 psi and temperature of 120-121^{0}C was maintained in autoclave. It was sterilized for 15-20 minutes and then cooled at room temperature.

4.4.3.Culturing: Culturing is a process in which a nutrient media is provided for micro-organism growth; the media is called culture media.

Culture media: Mineral salt Agar media

4.4.3.1. Isolation of Microorganisms capable of degradation of phenol:

After the cooling takes place, by using laminar flow hood (Product protection from microbial contaminants), an industrial effluent of about 1 ml was suspended in 10 ml of distilled water, stirred well for about 30 min and filtered. From this, 0.1 ml sample was pipetted out and surface spread in each petri plate containing nutrient agar, nutrient agar + 200 mg/l phenol including the petriplate containing mineral salt agar medium (MSAM). All the petriplates were incubated at 37^{0}C for about a week. Regular observations were made.

4.4.4.Selective Isolation of Phenol degrading Bacteria:

There are 6 isolated bacterial strains which are designated as DT-1, DT -2, DT -3, DT -4, DT -5and DT -6, among which DT -3 is proved to be more efficient in degrading phenol. Effluent sample was enriched in sterile Mineral salt agar medium (MSAM) using phenol as the sole source of carbon and further treated with phenol to ensure that only phenol resistant strain would be selected.

Screening and selection of phenol degrading microorganisms:

After incubation, the representative organisms growing on petriplates were purified. The pure microorganisms were tested for their ability to grow on phenol by inoculating to the MSAM media containing phenol(200ppm). The strains capable of growth at these concentrations were selected. After one week the well-defined colonies were purified by streaking on agar plates containing the same medium by streak plate method.

4.5. Study of Growth Kinetics:

4.5.1.Strain selection based on phenol acclimatization:

The isolated strains DT-1, DT -2, DT -3, DT -4, DT -5and DT -6,were inoculated into MSM containing phenol as carbon source for 48 hours shaking at 120rpm. After 48 hours, the Cell density was determined spectrophotometricallyby measuring turbidity at 600nm.

4.5.2. Phenol degrading studies:

The isolated strain DT-4 was grown in MSM medium by incubating overnight at 37°c on shaker at 120rpm. The 24 hrs old culture was inoculated into MSM medium with phenol as sole carbon source. Preliminary degrading studies were carried out with addition of isolated strain containing different concentrations of phenol, different periods of time, different pH values and different temperature conditions. The reaction mixture containing all components but devoid of isolated culture was used as control. The phenol concentrations were determined by analysing samples at every 24 hrs. interval by using UV Spectrophotometer. The residual amount of phenol present in the sample was measured by calorimetric assay 4- amino antipyrine method.

4.5.3. 4- Amino Antipyrine method:

When phenol reacts with 4- Amino Antipyrine in the presence of potassium ferricyanide forms coloured antipyrine dye this dye is kept in aqueous solution. Freshly inoculated culture of 5ml was taken by adding 95ml of phenol broth medium containing 1-3g/L phenol on 12 hours interval and then centrifuged at 1200 rpm for 1 hr. Supernatant was collected and sample was prepared for measurement of optical density of phenol. Phenol analysis was carried out by measuring at wavelength 500nm using UV spectrophotometer, after colour development by 4- Aminoantipyrine method forthe examination of sample.

- **Ammonium Hydroxide**: Dissolve 3.5 ml NH_4OH in 100ml Distilled water
- **Phosphate Buffer Solution:**Dissolve 64.5g K_2HPO_4, 72.3g KH_2PO_4 in distilled water and dilute to 1L. The pH should be 6.8
- **4-Amino antipyrine solution (AAP) :**Dissolve 2 grams of 4-Amino antipyrine in 100ml of dist. water
- **Pottasium ferricyanide solution:** Dissolve 8 grams of $K_3Fe(CN)$ in 100ml dist. water. Filter if necessary. Store it in a brown glass bottle. Prepare fresh weekly.
- **Sample mixture contains:** 0.9 ml distilled water + 0.1 ml supernatant sample + 2.5 ml of NH_4OH + 1ml of AAP + 1 ml of $K_3Fe\ (CN)_6$.

4.6. Phenol Standard Curve:

- Into a series of test tubes,1ml of phenol of various concentrations (0.1 to 0.5 ppm) was taken.
- To each test tubes,2ml of phosphate buffer solution was added and pH is adjusted
- To the above test tubes, 2.5ml of 0.5 N NH_4OH was added.
- To that 1ml of 4- Amino antipyrine solution was added.
- After 2 minutes, 1ml of Potassium ferricyanide solution was added.
- The readings were spectrophotometrically read at 500nm.
- A standard graph was constructed by taking phenol concentration (ppm) on X- axis and the corresponding absorbance on Y- axis. The values were tabulated in table and were shown in 3.3 and standard curve shown in the fig 3.2.

Table 4.3. Phenol standard curve data

Concentration	Absorbance(500nm)
0.0	0.0
0.1	0.635
0.2	1.171
0.3	1.680
0.4	2.312
0.5	2.793

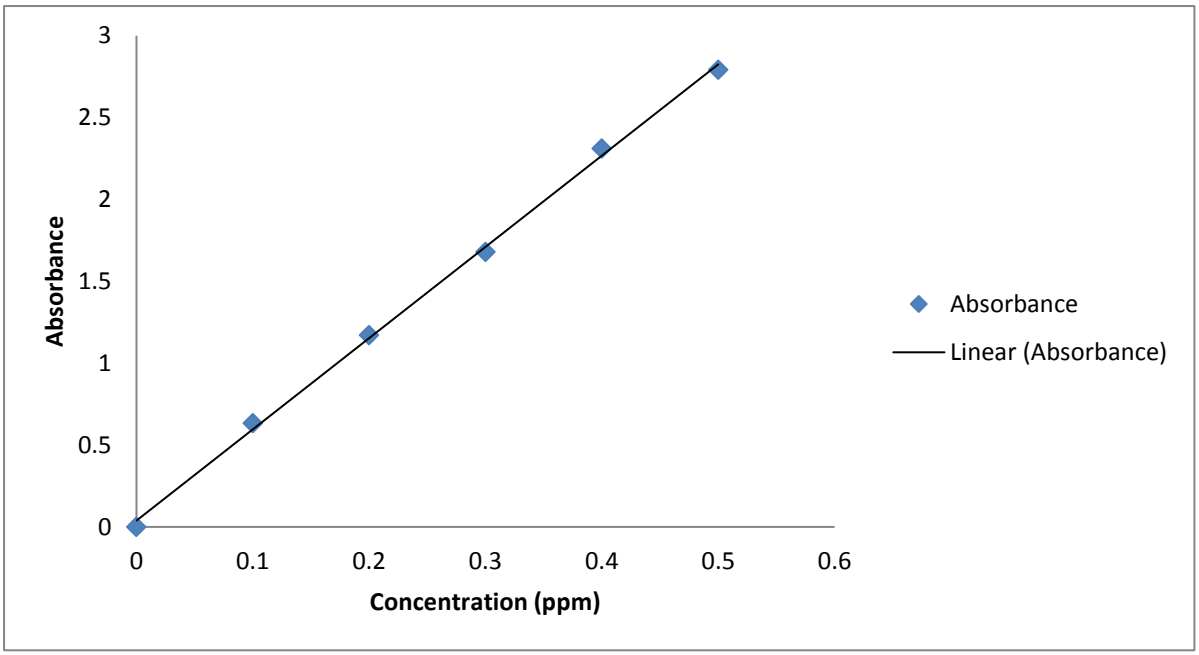

Fig 4.2. Phenol standard curve

4.7. Preliminary Studies for determining Optimum conditions:

Selected newly isolated bacterial strains were grown in the nutrient broth by incubation at overnight, at 37^0C on shaker at 120rpm. This 24 h young culture used tooptimize the following culture conditions of phenol biodegradation with inoculatedinto MSM medium which contained 200 mgL^{-1}phenol.

Optimization of the following physical and chemical parameters of phenoldegradation by selected newly isolated bacteria was carried out with MSM medium.Phenol degradation experiments were carried out in 250mL shake flask containing 100mL of MSM medium with 200mgL^{-1} of phenol as sole carbon source. The mediumwas inoculated with DT-4 strain to

initiate the cultivation and degradation of phenol and centrifuged at 1400rpm for 1 hr. The supernatant was withdrawn at a regular intervals (24hr) andanalyzed for cell growth and phenol concentration.

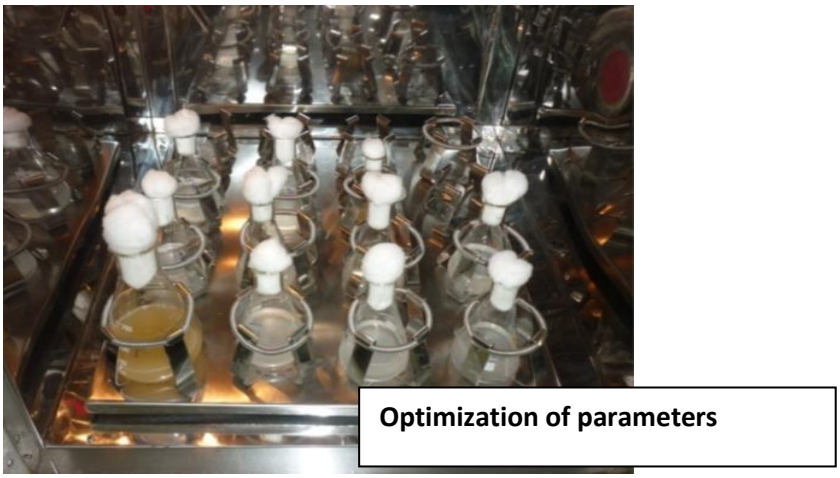

Figure 4.3. Optimization of parameters

4.7.1. Effect of contact time:

Effect of Contact time on degradation was studied at different period of times varying from 24h to 120h. All the prepared media were autoclaved and carefully inoculated with DT-4 strain culture using a micropipette and incubated in an orbital shaking incubator at 37°C and 120rpm. Sampling was performed for every 24 hours and the data obtained through spectrophotometric analysis were collected and converted into percentage degradationusing the 4- Amino antipyrine method.

4.7.2. Effect of initial phenol concentration:

Degradation activity of the culture of DT-4*Strain* was studied at different phenol concentrations varying from 200mg/l to 1400mg/l. All the prepared media were autoclaved and carefully inoculated with DT-4 strain culture using a micropipette and incubated in an orbital shaking incubator at 37°C and 120rpm. Sampling was performed for every 24 hours and the data obtained through spectrophotometric analysis were collected and converted into percentage degradation using the 4- Amino antipyrine method.

4.7.3. Effect of pH:

Effect of pH on degradation was studied at different pH varying from 4 to 12. pH of the media were adjusted using 0.1N HCL and 0.1N NaOH. All the prepared media were autoclaved and carefully inoculated with DT-4 strain culture using a micropipette and incubated in an orbital shaking incubator at 37°C and 120rpm. Sampling was performed for

every 24 hours and the data obtained through spectrophotometric analysis were collected and converted into percentage degradation using the 4- Amino antipyrine method.

4.7.4. Effect of temperature:

Effect of temperature on degradation was studied at four different temperatures (25, 28, 32, 36, 38 and 42). All the prepared media were autoclaved and carefully inoculated with DT-4 strain culture using a micropipette and incubated in an orbital shaking incubator at selected temperatures at 120 rpm. Sampling was performed for every 24 hours and the data obtained through spectrophotometric analysis were collected and converted into percentage degradation using the 4- Amino antipyrine method.

4.8. Determination of % phenol degradation:

The extent to which the test sample was degraded by the microorganism after finding out both the absorbance value in UV-Visible spectrophotometer and its corresponding value of phenol concentration from 4- Amino antipyrine method was calculated using the below given formula.

$$\% \text{ Phenol Degradation} = \frac{\text{Initial concentration-final concentration}}{\text{Initial concentration}} \times 100$$

4.9. Optimization of process parameters using Box- Behnken design:

For optimizing a process with 3 variables at 3 levels, Box- Behnken design of RSM is widely used. Range fixation for process variables is quite crucial when carrying experiments according to any statistical design of experiment, otherwise once the experimentation is over, the optimal conditions obtained by RSM might not be found within the chosen range. On the basis of preliminary studies, the range is fixed for the 3 chosen variables. The parameters selected for optimization by Box-Behnken include temperature(X1), contact time (X2) and concentration (X3). The three chosen variables are coded according to the relation:

$$X = (X-X_0)/\Delta X$$

Where x is coded variable, X is natural variable, X_0 is the middle point (zero level) and ΔX is the step change that represents the difference between the successive levels.

Box- Behnken design consists of 15 experimental runs representing the different combination of factors that are represented in coded form -1, 0, or +1. The runs 13, 14, 15 are

the replicates (repetitions) at the centre point and are used to estimate the experimental error are shown in Table 4.4.

$Y= \beta_0+ \beta_1 X1+ \beta_2 X_2+ \beta_3 X_3+\beta_{11}X_1 X_2+ \beta_{22}X_1 X_3 + \beta_{33} X_2 X_3+ \beta_{12} X_1^2+ \beta_{13} X_2^2+ \beta_{23} X_3^2$

Where, Y is the predicted response, $\beta_0$is the intercept, β_1, $\beta_2$and $\beta_3$are linear coefficients, β_{11} , $\beta_{22}$and β_{33} are the quadratic coefficients, $\beta_{12,}$ β_{23} and β_{13} is the interaction coefficient and X1, $X_{2,}$ $X_{3,}$ $X_1 X_{2,}$ $X_1 X_{3,}$ $X_2X_{3,}$ X_1^2, X_2^2 and $X_3^2$are independent variables. Using the 'MATLAB function regstats', the data obtained was analysed and response surface plots were constructed which indicated the possibility of degradation of phenol. Statistical analysis of the model was performed to evaluate the Analysis of Variance (ANOVA).

Table 4.4. Box- behnken matrix for optimization of 3 experimental factors in coded values

Runs	X1	X2	X3	Final response
1	-1	-1	0	-
2	-1	1	0	-
3	1	-1	0	-
4	1	1	0	-
5	-1	0	-1	-
6	-1	0	1	-
7	1	0	-1	-
8	1	0	1	-
9	0	-1	-1	-
10	0	-1	1	-
11	0	1	-1	-
12	0	1	1	-
13	0	0	0	-
14	0	0	0	-
15	0	0	0	-

4.10 Immobilization Of Microbial Strains To IncreaseThe Efficiency Of Phenol Biodegradation:Production Of Inoculums For Use In The Preparation Of Immobilized Cells:

Each microbial strain was inoculated in sterile nutrient broth and was incubated for24 hours at 30°C, 120 rpm in a 250 ml Erlenmeyer flask. The cells obtained from the mediawere used for immobilization in the Ca-Alginate beads.

4.11 Production Of Immobilized Cells:

Liquid cultures were centrifuged in a 50-ml plastic centrifuge tube (2,500 g) at room temperature for 10 min and the supernatant was discarded. The pellet was resuspended with previously autoclaved solution of sodium alginate to a final concentration of 4% (w/v) and10% (v/v) bacterial biomass. The alginate-bacterial mixture was added drop wise with sterile syringe (20 ml) fitted with a wide bore needle (1 mm diameter) from a height of about 20cm into an autoclaved solution of calcium chloride (3% (w/v), adjusted to pH 7.0), where beads formed immediately. The beads were left in this hardening solution overnight at 4°Cbefore being harvested by filtration.

4.12 Degradation Experiments And Analytical Methods:

Phenol degradation experiments were performed in shake flasks with immobilized in the same procedure as followed for the free cells as mentioned. Undegradedphenol left in the media was estimated by the method of based on rapid condensation with 4-amino-antipyrene as per the protocol.

4.13 A simple, straight forward procedure:

The identification of microorganisms in GEN III Biolog the process involves the following process as

- Isolated a pure culture of bacteria on agar media
- Prepared inoculum at specified cell density
- Inoculated the BiologMicroPlateovernight.
- Incubated the plate and observe the reaction pattern and entered the reaction pattern to obtain ID result.

Isolate

Prepare

Inoculate

Incubate and read

Fig 4.4. Microbial Identification with microbial identification system

4.14 Microbial Identification Databases For Biolog Systems:

Biolog Microbial Identification System is based on metabolic phenotypes. It is based on the theory that a species of bacteria develops a unique metabolic finger-print on a set of carbon sources and biochemicals. The cultured bacteria is tested for utilization of different carbon sources and biochemicals, which are pre-filled and dried into a 96 well test plate. Cells utilizing nutrient, respire and release energy which reduces proprietary Tetrazolium dye to form a distinct purple colour. Biolog data collection software is used to record the unique metabolic profileinto the computer which can be compared with thousands of profiles (corresponding to thousands of species) stored in the Biolog databases. If the profile is matched, computer displays the identified species.

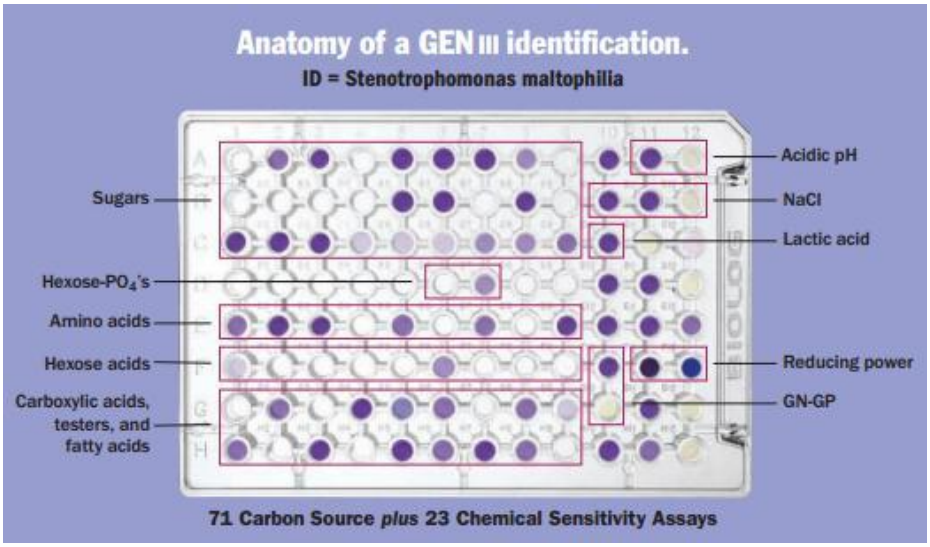

Fig 4.5. Anatomy of Gen III Identification

Biolog has designed proprietary microplates for identification of a wide range of microbesupto species level, such as Gen III plate (for gram negative and gram positive aerobic bacteria), AN plate (for anaerobic bacteria), YT plate (for yeast) and FF plate (for filamentous fungi). Nearly 2550 species are covered by Biolog for identification.

4.15 Gen III Microstation System

Micro station is a semi-automated microbial identification. It is Plate reader which is linked to a computer configured with software related to data collection and microbial identification software. It is capable of reading all types of BiologMicroplates. A microplate loaded with suspension of a test organism is incubated in a user-provided incubator and read

using Microstation. The metabolic finger print is read and sent to the computer for recording and eventual comparative studies with profiles already stored in Biolog databases. Computer reports the species/genus when the metabolic finger print is matched with those present in Biolog Database. The Micro Station™ is a versatile ID System and has the ability to characterize and identify distinguished range of pathogenic and environmental organisms among different areas of microbiology. Over 2650 species of bacteria, yeast and filamentous fungi can be identified using all Biolog databases, in as little as 2 hours.

Fig 4.6. Gen III Micro station System

Microlog M is the Manual Version of Biolog's Microbial Identification System. It uses the same Gen III plate and Gen III Microbial Identification software, as are used in automated systems. Difference is that Computer and Plate Reader, are not provided in Manual System. Microbial culture is used to inoculate the Gen III plate, which is then incubated in a lab incubator. Plate is read for all the 96 tests manually in terms of Positive or Negative reaction (If color appears, it is positive otherwise negative). The plate results (positive or negative), are fed manually in Gen III software which is provided and loaded in a user provided computer. Software does the analysis, searches Biolog provided database, and then reports the species. You can also update the database with those genus/species which are not present in Biolog database (using an optional Retrospect). **Manual system can be used for only aerobic bacteria**.

4.16 Phenotypic Microarray:

Phenotypic Microarray analysis feature is for the determination of cellular nutritional phenotypes in a wide range of microbial cells. A new isolated or an existing microbe, can be characterized for nearly 2000 cellular phenotypes which are preconfigured into 20 x 96 well arrays. These include tests for utilization of a wide variety of C, N, P, S sources (~800 tests), pH growth range (~100 tests), ionic and non-ionic osmotic sensitivity (~100 tests), and sensitivity to chemical agents (including anti-microbialsand anti-cancer compounds) that disrupt various biological pathways (~1,000 tests).Phentopic Microarray is successfully implemented with all the model bacteria such as Escherichia coli, Salmonella enterica, Pseudomonas aeruginosa,Pseudomonas putida, Sinorhizobiummeliloti, Saccharomyces cerevisiae,Bacillus subtilis, Bacillus cereus, Shewanellaoneidensis, Proteus mirabilis, and other species. Phenotypic Microarray technology is also available for mammalian cells.Phenotypic Microarray can be used to compare mutant cell with wild type cell. Phenotypic microarray is also used for General cell characterization microbes and also used for determine the metabolic properties of any microbe.

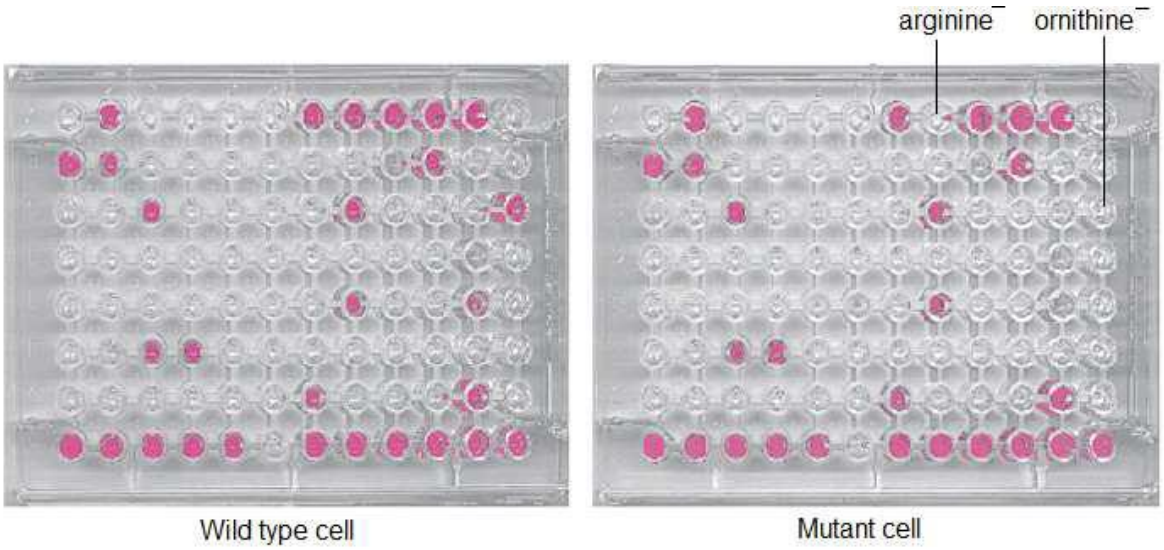

Fig. 4.7. Phenotypic Microarray

4.17 Growth on monoaromatic compounds:

Bacterial culture was inoculated in MSM media supplemented with 100 mg/l phenol and incubated in orbital shaker at 120 rpm, 37 ^{O}C for 72 hours. After 1.0 ml of the culture was transferred to a 1.5 ml micro centrifuge tube and centrifugedthe culture at 13,000 rpm for 5min to pellet the cells. The supernatant was discarded. The cell pellet was resuspended in 600 µl lysis buffer and vortex to completely resuspend cell pellet. Incubated for 1 h at 65 °C. An equal volume of chloroform and isoamyl alcohol were added at 24:1 ratio mix well by

inverting the tube until the phases are completely mixed and Spinned at 13,000 rpm for 5 min. The upper aqueous phase was carefully transferred to a new tube and repeated it until the white protein layer disappears. To precipitate the DNA 50 µl 3M Ammonium acetate was added. After that 2 volume of cold isopropyl alcohol was added. The tube was incubated at -20 °C for 30 min and spinned at13000 rpm for 10 min at 4 °C. The supernatant was discarded and the DNA pellet with 1 ml 70% ethanol was washed before spinning at max speed for 2 min. The supernatant was carefully discarded and the DNA pellet was air-dried. The DNA was resuspended in TE buffer. The isolated Gemonic DNA was checked on an agarose gel.

4.18 16S rRNA gene isolation and sequencing:

Genomic DNA was isolated from the bacterial strains that werecapable of degrading one or more of the monoaromatic com-pounds tested. For bacterial identification, the phenol degrader was incubated in MSM medium for 12h and the DNA was extracted. The 16S rRNA gene was amplified using bacterial universal primers 27f (50-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-30) and 907 R CCGTCAATTCMTTTRAGTTT. Purified DNA was then subjected to PCR amplification.

4.19 Sequence analysis:

The sequences were edited to remove vector contaminants and primer sequences. To identify the sequences, the clonedsequences were compared with the 16S rRNA gene sequencesof existing bacteria in the NCBI database. Related sequenceswere obtained from the GenBank Nucleotide database usingthe BLAST search program. All the sequences were editedto a common length and aligned using the ClustalW pro-gram. A phylogenetic tree was constructed by neighbor joiningmethod.

4.20 Taxonomic identification of the isolates:

On the basis of the consensus sequences for the 16S rRNAgene, a phylogenetic tree was constructed using sequencesfrom the isolates and representative bacteria.To test the stability of the groups, a bootstrap analysis of 10,000 replications was performed with a MEGA 6 version.

4.21 Amplification of dioxygenase genes:

The templates for PCR amplification were the genomic DNAthat was isolated from the bacteria that were used previously for 16S rRNA gene amplification. Genomic DNA was

extracted from the phenol-enriched culture that had the highest degradation rate. To study the phenol-degradation pathways, detection of the key functional genes in the biochemical pathway was performed via PCR using primers for phenol hydroxylase (Lph), catechol1,2-dioxygenase(1,2-CTD),catechol2,3-dioxygenase (2,3-CTD), and aromatic hydroxylase a-subunits (TBMD).

Table. 4.5. PCR Reaction Mixtures

Constituent	Initial Concentration	Volume (µL) for		Concentration in PCR mixture
		General detection	Bacteria detection	
10x PCR Buffer	200mM Tris-HCl 500mM KCl	2.5	2.5	20mM Tris-HCl 50mM KCl
$MgCl_2$	50mM	1	1	2mM $MgCl_2$
Taq DNA Polymerase	5U/µl	0.2	0.2	1 Unit
Dntp (Mix)	10mM	0.5	0.5	200 µM each dNTP
Primers Forward	Pmol/µL 10	0.3	0.4	0.12 µM for general detection 0.16 µM for bacterial detection
Reverse	Pmol/µL 10	0.3	0.4	0.12 µM for general detection 0.16 µM for bacterial detection
Template DNA	Variable	1	2	Variable
D>D> water (sterile)	-	19.2	18	-
Final Volume PCR mixtures		µL 25	µL 25	-

The genes encoding these enzymes were amplified by using the primers sets Lphf (5'-CGCCAGAACATTTATCGATC-3'), Lphr (5'-AGGCATCAAGATCACCGACTG-3') (Xu etal., 2001); 1,2-CTDf(5'-ACCATCGARGGYCCSCTSTAY-3'), 1,2- CTDr (5'-GTTRATCTGGGTGGTSAG-3'); 2,3-CTDf (5'-GARCTSTAYGCSGAYAAGGAR-3'), 2,3-CTDr (5'-RCCGCTSGGRTCGAAGAARTA-3') (Sei and Fathepure, 2009); and TBMDf (5'-CGCCAGAACCACTTGTCRRTCCA-3'), TBMDr (5'-ACC GGGATATTTYTCTTCSAGCA-3') (Hendrickx et al., 2006). PCR amplifications were performed using a 25-µL mixture of 12.5 µL PCRTaq-mix, 0.5 µL of each primer, 2 µL of template and 8.5 µL of ddH2O. The amplification conditions were as follows: initial denaturation at 95^{O}C for 5 min; 35cycles of 95^{O}C for 50s, 56^{O}C for 30s, and 72^{O}C for1:30 min; and a final extension phase at 72^{O}C for 5 min.

Table 4.6.Conditions for universal detection of phenol-degrading bacteria

Steps	Sub-Step	Time (min)	Temperature (^{0}C)
Initial Denaturation	-	10	94

Step 1 (5 Cycles)	Denaturaton	1	94
	Annealing	1	58
	Extension	1	72
Step 2 (5 Cycles)	Denaturaton	1	94
	Annealing	1	57
	Extension	1	72
Step 3 (5 Cycles)	Denaturaton	1	94
	Annealing	1	56
	Extension	1	72
Final Extension	-	10	72

4.22 Nucleotide sequence accession numbers:

The nucleotide sequences obtained in this study were deposited in the GenBank Nucleotide database in NCBI.

After several weeks of enrichment, the bacterial strain capable of growing on phenol of higher concentrations as sole carbon and energy source, the DNA was isolated. The potent strain named DT-4 was eventually selected for further studies.

4.23 16S rRNA gene isolation and sequencing analysis:

16S rRNA gene sequencing has been established as the gold standard for identification and taxonomic classification of bacterial species. The hyper variable regions of 16S rRNA gene sequences provide species-specific signature sequences useful for bacterial identification. In this work, comparison of the bacterial 16S rRNA sequence has been emerged as a valuable genetic technique and can lead to the recognition of novel bacterial enzymes in *Pseudomonas* species. The sequence of the phenol degrading bacteria *Pseudomonas syringae pv maculicola*is

4.24 Determination of Kinetic Parameters:

Using the microorganism concentration values during the exponential growth phase, the values for μ were obtained. Haldane equation has shown by several researchers to give an adequate fit to plot of μ versus S. The model equations were solved with the aid of MATLAB 7.4 software for the nonlinear regression method. The model fitted with the data and also estimated the values of three biokinetic constants of Haldane equation. The dependence of specific growth rate on phenol concentration is shown in Figure 2. From this plot, the specific growth rate was increased with an increase in the initial phenol concentration to a certain level and then a decrease was started with a more increase in the concentration.

5. Results and Discussion

The present study deals with the isolation of phenol degrading bacteria with potential to degrade phenolic compounds which is one of the most alarming situation in today world by the generation of a huge amount of waste water contaminated with the toxic organic substances like phenolics from the industrial sector. In the present investigation, attempts were made to isolate phenol degrading microorganisms from industrial effluents and enrichment to select the most efficient strain for phenol biodegradation.

5.1. Fourier Transform Infrared Spectroscopy (FTIR) analysis:

FTIR analysis was carried out using Bruker FTIR (accessory-ALPHA; software: opus.6.4) at a resolution of 6 cm^{-1} and the changes in % transmission at different wavelengths were observed. In the FTIR analysis, the bands located within the range were due to O–H stretch, H–bonded respectively. The spectra obtained after detection were in the region 3200-3500 cm^{-1}which indicates the presence of phenols (Figure 5.1).

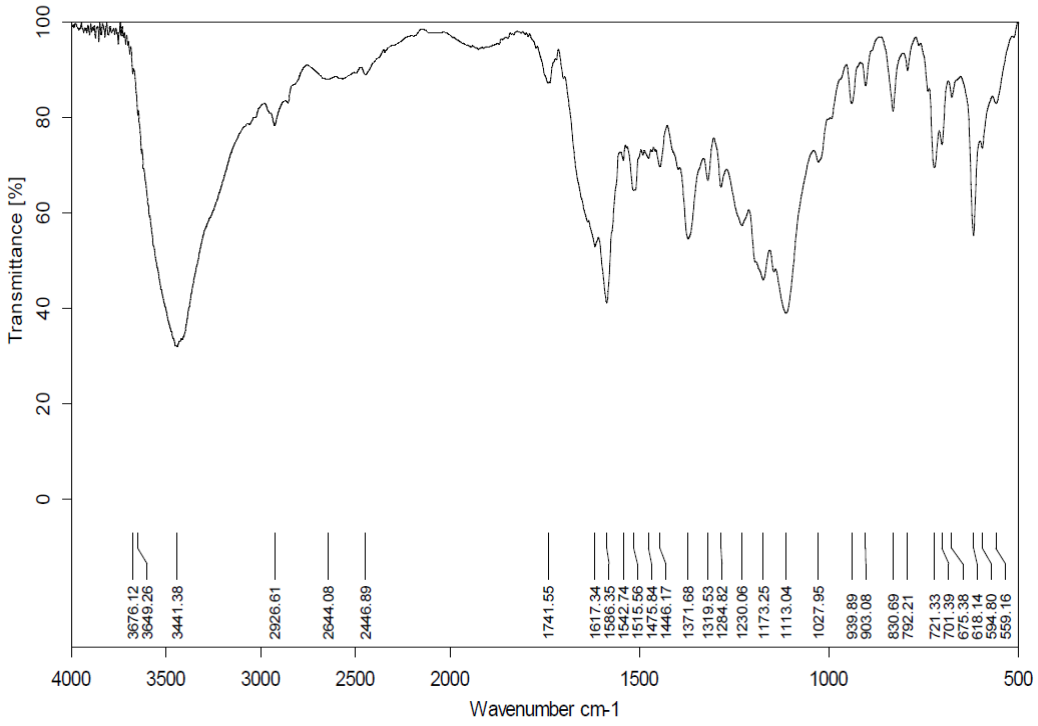

Figure 5.1. FTIR Spectrum of the sample

5.2. Steam distillation:

The steam distillate of 100ml was collected andthe collected distillate was analysed for phenols byusing 4- amino antipyrene method. The concentration of phenol obtained was 46ppm.

5.3. Isolation and characterization of bacterial strains:

5.3.1. Selective Isolation of Phenol degrading Bacteria:

There are 6 isolated bacterial strains which are designated as DT-1, DT-2, DT-3, DT-4, DT-5and DT-6. The Performance of each of these bacterial strains is evaluated and their biodegradation rate was observed. Among the isolated strains, DT-4 is proved to be more efficient in degrading phenol.

Effluent sample was enriched in sterile Mineral salt agar medium (MSAM) using phenol as the sole source of carbon and energy. The sample was further treated with phenol to ensure that only phenol resistant strain would be selected. Upon enrichment with a xenobiotic compound, the natural selection of microorganisms adapted to the presence of a xenobiote has high potential for the biodegradation of the compound. Six isolates that were able to utilize phenol as sole source of carbon were obtained from the enriched population grown in MSAM medium, supplemented with phenol. These six were selected which yielded more than 80% phenol degradation and they were subjected to higher initial phenol concentration like 500 ppm and 1000 ppm. Of the strains tested, DT-4 showed a higher potential to degrade phenol at both 500 and 1000 ppm (Figure 5.2).

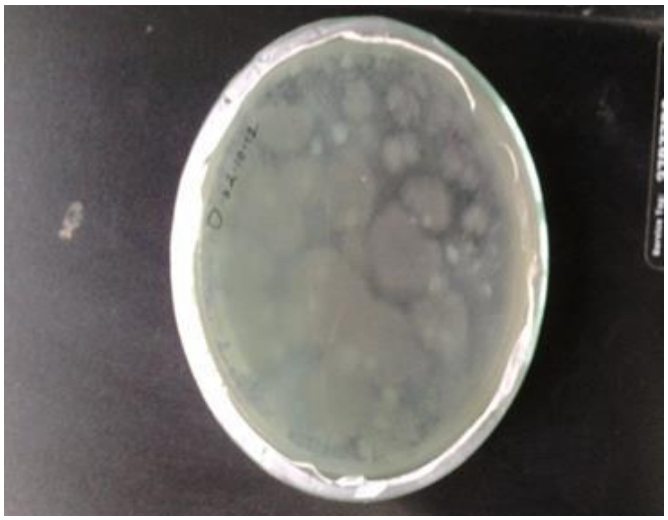

Fig 5.2.Isolated bacterial culture

5.3.2. Selection of phenol degrading microorganisms:

Pure strains capable of degrading phenol were selected by growing on MSAM medium plates by streak plate method (Figure 5.3).

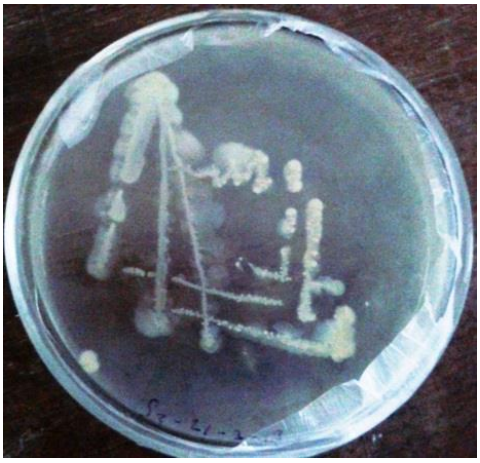

Fig 5.3.Streak plate method

5.3. Optimization of physiological parameters (Contact time, Concentration Temperature and pH):

Growth and biodegradation of any microorganism depends on various physiochemical parameterslike contact time, temperature of incubator and pH of the medium and concentration of phenol which is used as a sole of carbon source and energy. Adsorption of any substance is also influenced by various parameters like dosage of carbon, temperature, pH, and concentration of phenol. Aim of this project, is to optimize these parameters. Study on phenol biodegradation and adsorption at different Contact time, concentration of phenol, temperature and pH and are carried out and optimize conditions are found out.

5.3.1. Effect of Contact time:

Degradation of Phenol at various periods of time by the isolated DT-4 strainwas studied. It was observed that the percentage of degradation was increased from 0 to 75 with an increase in contact of time from 24 hours to 120 hours These results show that the contact time of the medium is also an important factor with regard to degradation. The rate of degradation tends to decrease rapidly after 96 hours.

Influence of exposure time on the response of pure cultures of bacteria and microbial community to toxicity of phenol was assessed. At sufficient concentrations, exposure of these bacterial cells to phenol resulted in efficient degrading activity. Increase in exposure time resulted at lower concentrations of phenol. The toxicity threshold concentrations of phenol vary among the bacterial strains and the exposure time and indicate that bacteria could acclimate to phenol with increase in exposure time. It is suggested that for degradation of phenol is reliable and reproducible result would be best achieved within 96 h Similar results

were observed in the bacterial degradation of exposure time on phenol toxicity to refinery wastewater bacteria(C. O. Nweke et al., 2010)

5.3.2. Effect of initial Phenol concentration:

Experiments were conducted to study the effect of initial phenol concentration (200mg/l to 1200mg/l) on phenol removal from the solution. The results obtained are shown in Figure 5.5. The obtained plot shows that the percentage of degradation was decreased with an increase in initial concentration of dye. The percentage of degradation of phenol by isolated DT-4 strain was decreased from 77.7 to 61.7 with anincrease in initial concentration from 200 to 1200 mg/l.

The higher concentration of phenol inhibits nucleic acid biosynthesis and cell growth , so the effect of dye concentration on growth of organisms is an important consideration for its field application, (Khehra et al.2005) suggested that the decrease in degradation efficiency might be due to increase in the toxic effect of Phenol, with increase in Phenol concentration from 200 mg/l to 1200 mg/l.

5.3.3. Effect of temperature:

In microorganisms the environmental temperature establishes a direct relationship with microbial activity as the microbial cell, responds to temperature changes by adaptation via biochemical or enzymatic mechanisms. Experiments were conducted to determine the effect of temperature on percentage of degradation of Phenol with a constant 96 hrs time at different temperatures (25 to 42^{o}C) with initial phenol concentration of 200ppm. It was observed that Phenol degradation activity of the culture was found to increase with an increase in incubation temperature from 25 to 36 ºC with maximum activity attained at 36ºC (94.23% degradation). Cells may become metabolically active and capable enough to produce the required enzymes needed for degradation. Further increase in temperature resulted in marginal reduction in degrading ability of the bacterial culture. This might have occurred due to adverse effect of high temperature on theenzymatic activities (Cetin and Donmez, 2006). Similar results were observed in the bacterial degradation of Phenolic Compounds.

5.3.4. Effect of pH:

Degradation of phenol at various pH values by the strainwas studied. It was observed that the percentage of degradation was increased from 69.23 to 97.05 with an increase in pH from 2 to 8 and decreased from 97.05 to 92.13 with an increase in pH from 8 to 12. These

results show that the pH of the medium is also an important factor with regard to degradation. The rate of colour removal is higher at the optimum pH-8, and tends to decrease rapidly above pH-8. From the graph it is observed that the bacterium showed maximum degradation ability at pH-8. Degradation ability of bacteria depends on cell growth and active metabolism of culture. According to the results the organism used actively degraded the phenol at neutral alkaline conditions. By this study it can be understood that *the* strainactively grows at pH-8, consequently showing maximum phenol degradable ability at that pH. Similar results were observed in the bacterial degradation of various Compounds (Gurulakshmi et al.,2008).

5.4. Optimization of process parameters using Box-Behnken method:

The preliminary studies resulted in the following values of different process variables for maximum biodegradation of phenol (Table 5.1).

Table 5.1. Optimum parameter values obtained in preliminary studies

Parameter	Optimized value
Contact time	96hrs
Temperature	36ºC
Concentration	200ppm
pH	8

Three parameters were selected for optimization by Box- Behnken method which showed significant effect on biodegradation of phenol. They include

- Temperature of incubation (X_1)
- Contact time(X_2)
- Concentration(X_3)

The optimum values used in preliminary studies have been used as the basis for selecting the mid points(zero level) in Box- Behnken method (Table 5.2) for further optimization.

Table 5.2.coded and real values of medium components used for Box- Behnken

Independent Variables	Coded factors		
	-1	0	1

Temperature (ºC)	32	36	38
Contact time (hrs)	48	96	120
Concentration (ppm)	0	200	400

5.4.1. Experimental design for optimization:

Experiments were performed according to the Box- Behnken method given in Table 5.3in order to evaluate the optimum combination of selected components in the medium.

Table 5.3. Result of Box- Behnken for 3 factors and comparision of experimental and predicted values of Phenol biodegradation

Run No.	Temperature (ºC)	Contact Time(hrs)	Concentration (ppm)	% Degradation of phenol (Y)		
				Experimental	Predicted	$Y_{calculated}$
1.	32(-1)	48(-1)	200(0)	-4.017	-3.858	63
2.	32(-1)	120(+1)	200(0)	-2.882	-2.848	84
3.	38(+1)	48(-1)	200(0)	-3.194	-3.229	79
4.	38(+1)	120(+1)	200(0)	-3.442	-3.601	72
5.	32(-1)	96(0)	0(-1)	-3.576	-3.751	69
6.	32(-1)	96(0)	400(+1)	-3.507	-3.524	70
7.	38(+1)	96(0)	0(-1)	-3.863	-3.845	65
8.	38(+1)	96(0)	400(+1)	-3.730	-3.554	68
9.	36(0)	48(-1)	0(-1)	-3.963	-3.947	64
10.	36(0)	48(-1)	400(+1)	-4.200	-4.341	62
11.	36(0)	120(+1)	0(-1)	-4.423	-4.281	59
12.	36(0)	120(+1)	400(+1)	-3.352	-3.369	76
13.	36(0)	96(0)	200(0)	-2.465	-2.465	91
14.	36(0)	96(0)	200(0)	-2.465	-2.465	91
15.	36(0)	96(0)	200(0)	-2.465	-2.465	91

Using the results of the experiments, the following second order regression equation, giving degradation of phenol as a function of temperature (X_1), contact time (X_2) and concentration(X_3) was obtained. Using MATLAB function 'regstats', the following coefficients are estimated.

$$\mathbf{Y} = -2.4651 - 0.030905\,X_1 + 0.15936X_2 + 0.12957X_3 - -0.3457X_1X_2 - 0.016135X_1X3 + 0.32671X_2X_3 - -0.30154X_1^2 - 0.61735X_2^2 - 0.61735X_3^2 \quad (5.1)$$

The estimated coefficients along with their p-values were reported in Table 5.4

Table 5.4.Regression data for the model

	Coefficient	**Regression**	**Std. error**	**t- value**	**p- value**
constant	β_0	-2.4651	0.10198	-24.173	2.2579e-006
X_1	β_1	-0.030905	0.062448	-0.49489	0.64165
X_2	β_2	0.15936	0.062448	2.5519	0.051145
X_3	β_3	0.12957	0.062448	2.0749	0.092663
X_1X_2	β_{11}	-0.3457	0.088315	-3.9145	0.011244
X_1X_3	β_{22}	0.016135	0.088315	0.18269	0.86221
X_2X_3	β_{33}	0.32671	0.088315	3.6994	0.014009
X_1^2	β_{12}	-0.30154	0.091921	-3.2805	0.021943
X_2^2	β_{13}	-0.61735	0.091921	-6.7161	0.0011085
X_3^2	β_{23}	-0.61735	0.091921	-9.814	0.00018704

The coefficients of the regression model (Eq. 5.1) calculated were listed in Table 5.8. The significance of the each coefficient in equation (5.1) was determined by student's t-test and p- values which were also listed in Table 5.4. The larger the magnitude of the t- value and smaller the p- value, the more significant is the corresponding coefficient. The p- values were used as a tool to check the significance of each of the coefficients, which, in turn, are necessary to understand the pattern of the mutual interactions between the test variables(Khuri and Cornell, 1996). This implies that the linear, quadratic and interaction

effects of temperature, contact time and concentration were highly significant as is evident from their respective p- values.

The plot showed a satisfactory correlationbetween the experimental and predicted values of phenol degradation, wherein, the points clusteraround the diagonal line indicated the optimal fit of themodel, since the deviation between the experimental and predictedvalues was minimal.

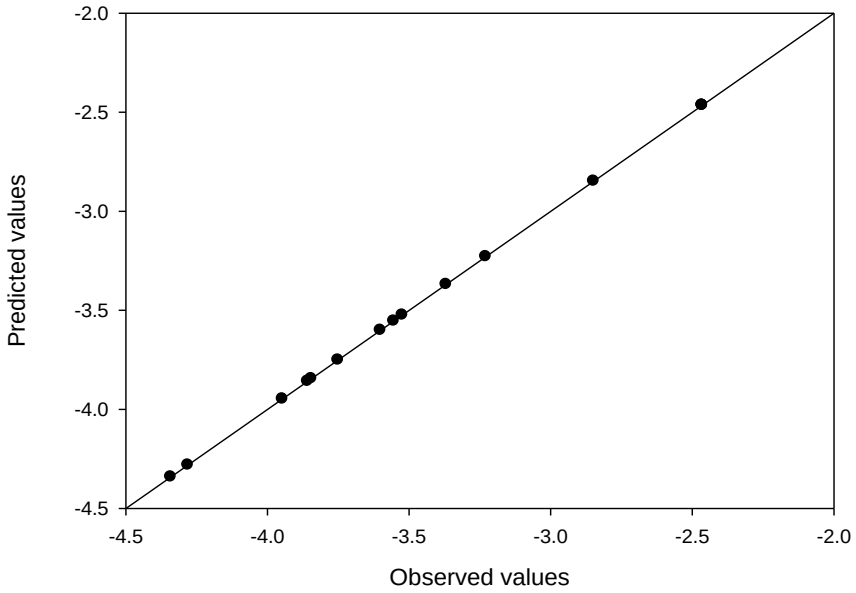

Figure 5.4. Comparison plot between experimental and predicted values of phenol degradation

The results of the second order response surface model fitting in the form of Analysis of Variance (ANOVA) and the value of the determination coefficient (R^2= 0.97247) were given in Table 5.9. It is required to test the significance and adequacy of the model. The Fisher variance ratio, the F- value ($=S^2_r/S^2_e$) is a statistically valid measure of how well the factors describe the variance in the data about it mean. The greater the F- value from unity, the more certain it is that the factors explain adequately the variation in the data about its mean, and the estimated factor effects are real. The ANOVA of the regression model demonstrates that the model is highly significant, as is evident from the Fisher's F- test (F_{model}= 19.6249) and a very low probability value(P_{model}= 0.0022).

Table 5.5. ANOVA for the model

Source of variations	Degree of freedom	Sum of squares	Mean square	F value	P value
Regressions	9.0000	5.5103	0.6123	19.6249	0.0022
Residual	5.0000	0.1560	0.0312		
Total	14.0000	5.6663			

The goodness of the fit of the model was checked by the determination coefficient (R^2). The R value provides a measure of how much variability in the observed response values can be explained by the experimental variables and their interactions. The R^2 value is always between 0 and 1. The closer the R^2 value is to 1, the stronger the model is and the better it predicts the response. In this case, the value of the determination coefficient (R^2= 0.97247) indicates that 97.24% of the variability in the response could be explained by the model.

5.4.2. Response Surface Plots:

The biodegradation of phenol over different combinations of independent variables was visualized through three- dimensional view of response surface plots in Response surface plot is a function of two factors at a time maintaining all other factors at a fixed level (zero for instance) which is more helpful in understanding both the main and interaction effects of the two factors. All the response surface plots revealed that at low and high levels of variables the degradation of phenol was maximal, however, there existed a region where neither an increasing nor a decreasing trend in the degradation of phenol was observed. This phenomenon confirmed that there was an existence of optimum for the fermentation variables in order to maximize degradation of phenol.

5.4.2.1. Effect of temperature and contact time on biodegradation of phenol:

The interaction effect of temperature and contact time on phenol degradation in Fig. 5.5clearly indicates a proper combination of, degradation of phenol. An increase temperature with contact timeincreased the degradation of phenol gradually but at a higher temperature with contact time the trend is reversed. The optimum formaximum phenol degradation lies near the centre point of the temperature and contact time.

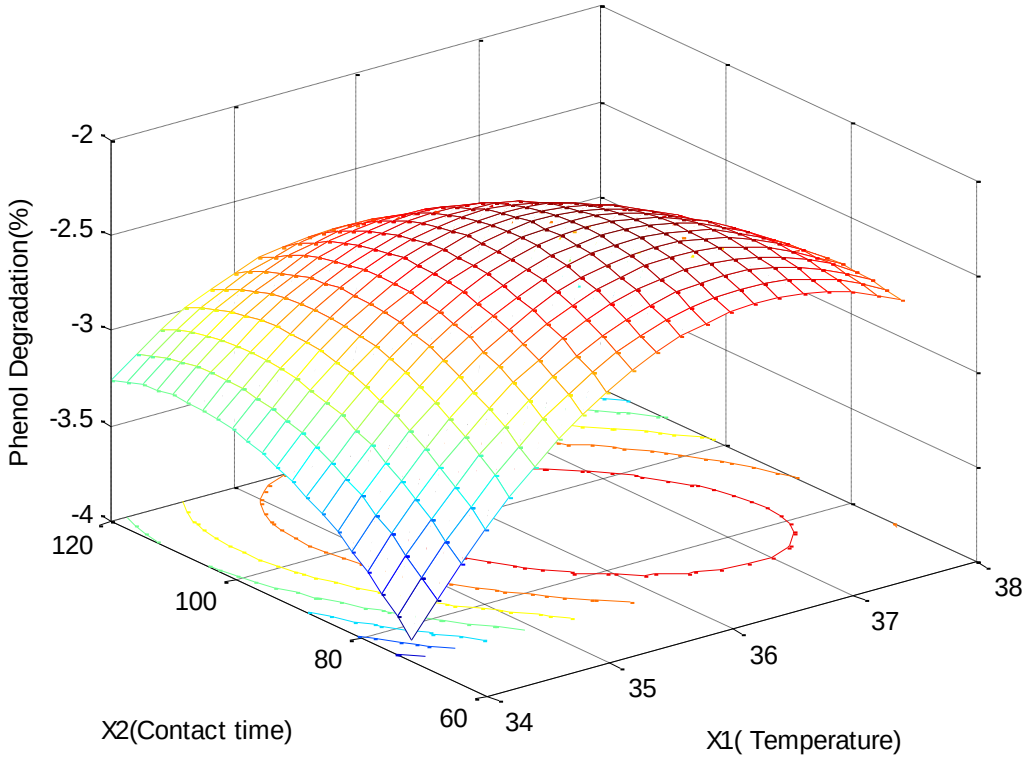

Figure 5.5.Response surface counter plot showing the effect of temperature and contact time on degradation of phenol

5.4.2.2. Effect of temperature and concentration on biodegradation of phenol:

A similar effect on the response was observed for temperatureand concentration. An increase in the temperature with concentration up to the optimum point increased the degradation of phenol to maximum level and a further increase in temperature with concentration decreased the degradation of phenol.

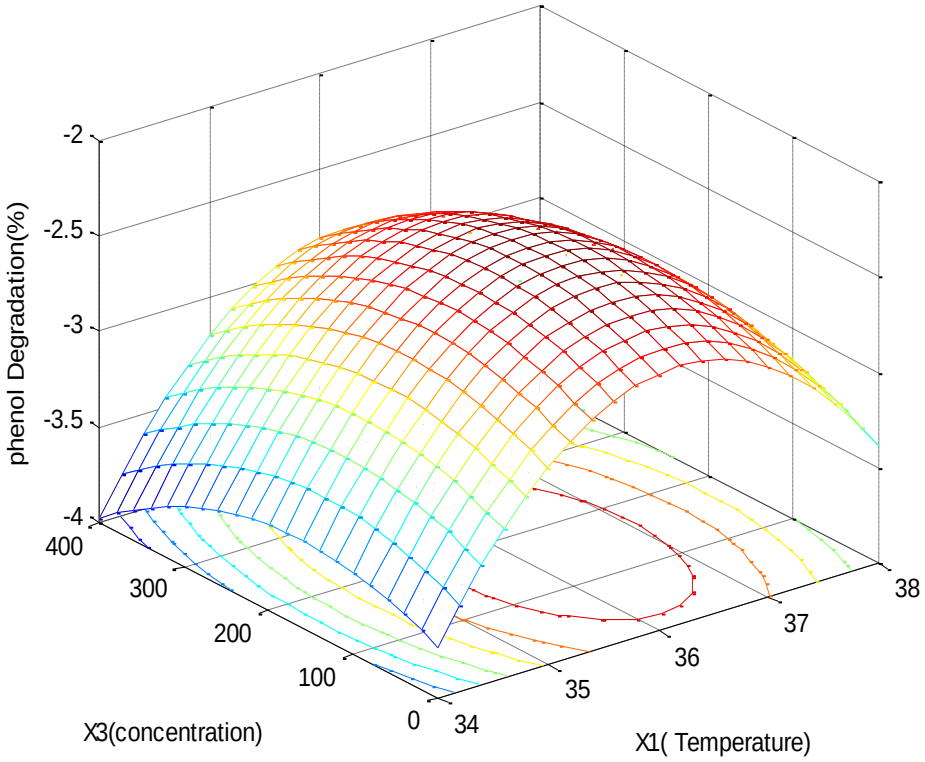

Figure 5.7. Response surface counter plot showing the effect of temperature and concentration on degradation of phenol

5.4.2.3. Effect of contact time and concentration on biodegradation of phenol:

The interaction effect of contact time and concentrationon phenol degradation in clearly indicates a proper combination of, degradation of phenol. An increase in the contact time with concentrationincreased the degradation of phenol gradually but at a higher contact time with concentration the trend is reversed. The optimum formaximum phenol degradation lies near the centre point of thecontact time and concentration.

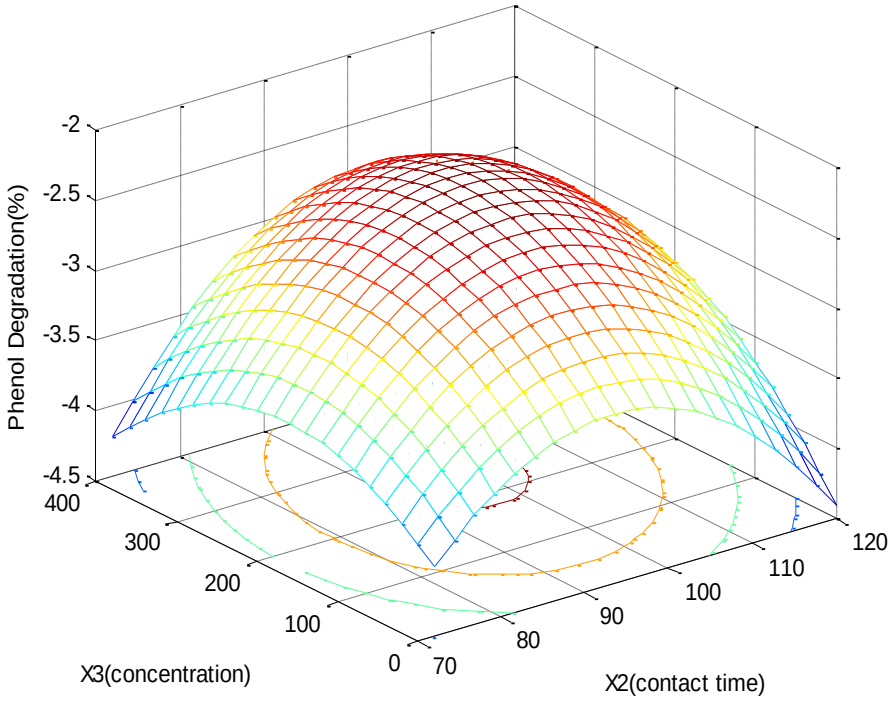

Fig 5.8.Response surface counter plot showing the effect of contact time and concentration on degradation of phenol

Therefore, an optimum was observed near the central valueof temperature, moisture content, contact time and concentration. The optimum conditions for maximum phenol degradation was obtained at temperature of35.6° C, contact time of100.8 hrs,concentration of 221 ppm. Anexperimentalphenol degradation of 0.0872 i.e. 91% was obtained at these optimum parameters. The experimental and predicted degradation of phenol at optimum conditions was shown in Table 5.10.

Table 5.6.Experimental and predicted degradation of phenol obtained from optimized parameters

Variable	**Codes**	**Natural scale**	**Optimum degradation of phenol**	
Temperature	-0.16513	35.66974	Experimental	Predicted
Contact time	0.20368	100.8882	0.0872	0.0865
Phenol concentration	0.10722	221.4441		

Log of optimum value of dependent variable= -2.4394

Eigen values = -0.98629 -0.6148 -0.21992

All the eigen values obtained in the present work are negative and hence, the nature of the response surface is maximum which is evident from the 3-D response plots in which the interactive effect of the 2 variables (with the third variable being fixed at its optimum value) on phenol degradation is depicted.

5.5.Immobilization Of Microbial Strains To IncreaseThe Efficiency Of Phenol Biodegradation: (Optimization of process parameters using Box-Behnken method)

5.5.1 Optimum parameter values obtained in preliminary studies:

The preliminary studies resulted in the following values of different process variables for maximum biodegradation of phenol (Table 5.7).

Table 5.7. Optimum parameter values obtained in preliminary studies (Immobilized Strains)

Parameter	Optimized value
Contact time	48hrs
Temperature	34ºC
Concentration	200ppm
pH	7

The optimum values used in preliminary studies have been used as the basis for selecting the mid points(zero level) in Box- Behnken method (Table 5.8) for further optimization.

5.8. Coded and real values of medium components used for Box- Behnken:

Independent Variables	Coded factors		
	-1	0	1
Temperature (ºC)	32	34	36
Contact time (hrs)	24	48	96
Concentration (ppm)	0	200	400

5.5.3 Experimental design for optimization:

Experiments were performed according to the Box- Behnken method given in Table 5.9 in order to evaluate the optimum combination of selected components in the medium.

Table 5.9 Result of Box- Behnken for 3 factors and comparision of experimental and predicted values of Phenol biodegradation

Run No.	Temperature (°C)	Contact Time(hrs)	Concentration (ppm)	% Degradation of phenol	
				Experimental	Experimental
1.	32(-1)	24(-1)	200(0)	-4.135	-4.135
2.	32(-1)	72(+1)	200(0)	-3.194	-3.194
3.	36(+1)	24(-1)	200(0)	-3.297	-3.297
4.	36(+1)	72(+1)	200(0)	-3.576	-3.576
5.	32(-1)	48(0)	0(-1)	-4.269	-4.269
6.	32(-1)	48(0)	400(+1)	-3.576	-3.576
7.	34(+1)	48(0)	0(-1)	-4.017	-4.017
8.	36(+1)	48(0)	400(+1)	-3.863	-3.863
9.	34(0)	24(-1)	0(-1)	-4.200	-4.200
10.	34(0)	24(-1)	400(+1)	-4.711	-4.711
11.	34(0)	72(+1)	0(-1)	-4.962	-4.962
12.	34(0)	72(+1)	400(+1)	-3.576	-3.576
13.	34(0)	48(0)	200(0)	-2.781	-2.781
14.	34(0)	48(0)	200(0)	-2.781	-2.781
15.	34(0)	48(0)	200(0)	-2.781	-2.781

Using the results of the experiments, the following second order regression equation, giving degradation of phenol as a function of temperature (X_1), contact time (X_2) and concentration(X_3) was obtained. Using MATLAB function ‘regstats’, the following coefficients are estimated.The estimated coefficients along with their p-values were reported in Table 5.10

Table.5.10Regression data for the model:

	Coefficient	Regression	Std. error	t- value	p- value
constant	β_0	-2.7806	0.052276	-53.191	4.4409e-008
X_1	β_1	0.052574	0.032013	1.6423	0.16145
X_2	β_2	0.12939	0.032013	4.0418	0.009905
X_3	β_3	0.21535	0.032013	6.7269	0.0011003
$X_1 X_2$	β_{11}	-0.30492	0.045273	-6.7353	0.0010941
$X_1 X_3$	β_{22}	-0.13475	0.045273	-2.9764	0.030928
$X_2 X_3$	β_{33}	0.47428	0.045273	10.476	0.00013671
X_1^2	β_{12}	-0.16956	0.047121	-3.5984	0.015569
X_2^2	β_{13}	-0.60025	0.047121	-12.738005	5.3022e-
X_3^2	β_{23}	-0.98103	0.047121	-20.819006	4.7347e-

The results of the second order response surface model fitting in the form of Analysis of Variance (ANOVA) and the value of the determination coefficient were given in Table 5.11

Table.5.11ANOVA for the model

Source of variations	Degree of freedom	Sum of squares	Mean square	F value	P value
Regressions	9.0000	6.4598	0.7178	87.5482	0.0001
Residual	5.0000	0.0410	0.0082		
Total	14.0000	6.5008			

5.5.4 Response Surface Plots:

The biodegradation of phenol over different combinations of independent variables was visualized through three- dimensional view of response surface plots in Response surface plot is a function of two factors at a time maintaining all other factors at a fixed level (zero for instance) which is more helpful in understanding both the main and interaction effects of the two factors. All the response surface plots revealed that at low and high levels of variables the degradation of phenol was maximal, however, there existed a region where

neither an increasing nor a decreasing trend in the degradation of phenol was observed. This phenomenon confirmed that there was an existence of optimum for the fermentation variables in order to maximize degradation of phenol.

5.5.4.1 Effect of temperature and contact time on biodegradation of phenol:

The interaction effect of temperature and contact time on phenol degradation in Fig. 5.5 clearly indicates a proper combination of, degradation of phenol. An increase temperature with contact timeincreased the degradation of phenol gradually but at a higher temperature with contact time the trend is reversed. The optimum formaximum phenol degradation lies near the centre point of the temperature and contact time.

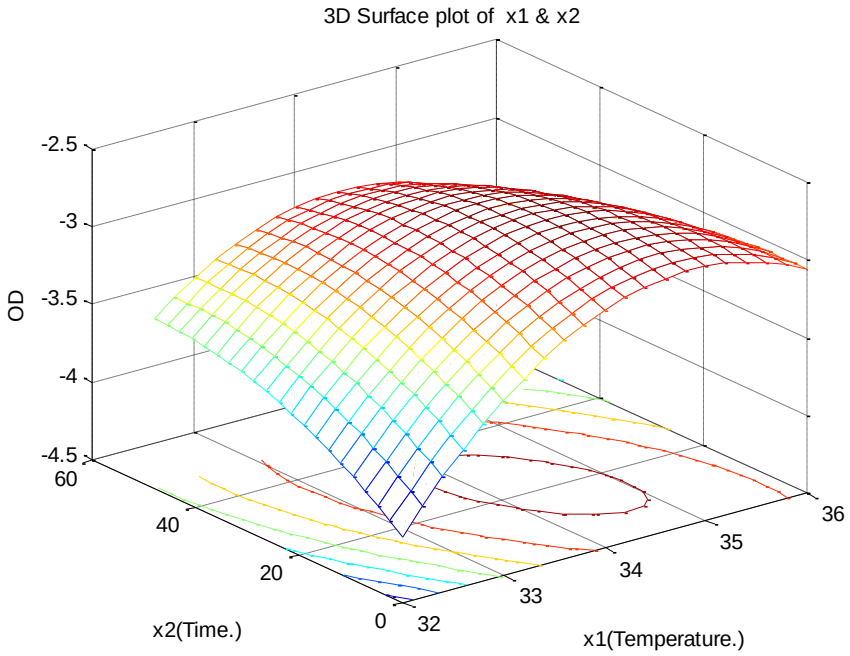

Fig. 5.9 Response surface counter plot showing the effect of temperature and contact time on degradation of phenol

5.5.4.2Effect of temperature and concentration on biodegradation of phenol:

A similar effect on the response was observed for temperatureand concentration. An increase in the temperature with concentration up to the optimum point increased the degradation of phenol to maximum level and a further increase in temperature with concentration decreased the degradation of phenol

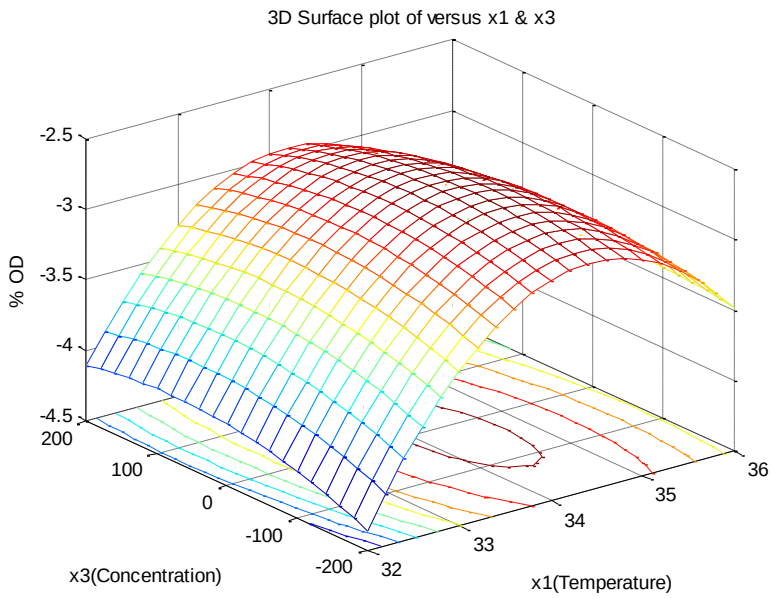

Fig. 5.10 Response surface counter plot showing the effect of temperature and concentration on degradation of phenol

5.5.4.3 Effect of contact time and concentration on biodegradation of phenol:

The interaction effect of contact time and concentrationon phenol degradation in clearly indicates a proper combination of, degradation of phenol. An increase in the contact time with concentrationincreased the degradation of phenol gradually but at a higher contact time with concentration the trend is reversed. The optimum formaximum phenol degradation lies near the centre point of thecontact time and concentration.

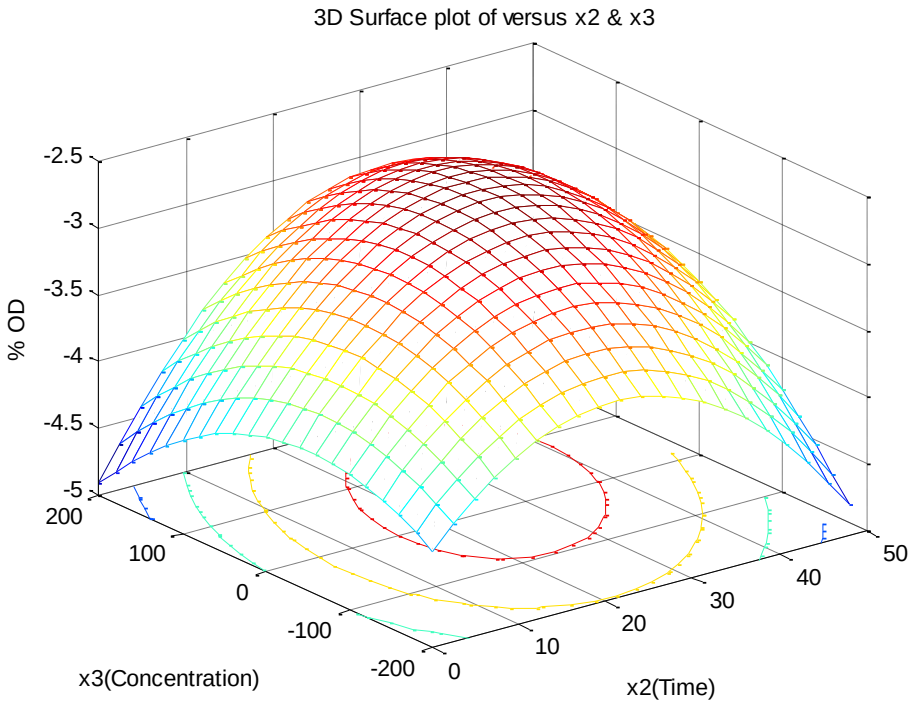

Fig. 5.11 Response surface counter plot showing the effect of contact time and concentration on degradation of phenol

5.6Experimental and predicted degradation of phenol obtained from optimized parameters:

An optimum was observed near the central value of temperature, moisture content, contact time and concentration. The optimum conditions for maximum phenol degradation were obtained at temperature of 33.8° C, contact time of 52.6 hrs, concentration of 232 ppm. An experimental phenol degradation of 96% was obtained at these optimum parameters.

Log of optimum value of dependent variable = -2.7529

Eigen values = -1.0948 -0.55798 -0.098078

5.7Validationof the model:

Thevalidationwascarriedoutinshake flasks underoptimumconditionsofthemediapredictedbythemodel. The experimental values for degradation of phenol were closer to the predicted values, thereby validating the model. So the validity of the equation and hence the response surface methodology are justified.

5.8 Identification of capable phenol degrading microorganisms, DT-4:

An appropriate BiologGenIIIMicroplate was used to actuate the relative capacity of substrate utilization of strain DT-4. The result interpreted that the isolate reacts substantially

with 39 of the 95 carbon substrates for 24 h of incubation. According to Biolog GN identification, the reaction profile was similar to that of *Pseudomonas syringaepvmaculicola* with maximum comparability.

Date & Time of Read	Nov 18 2016 1:34 PM
Biolog ID DB	GEN_III_v2.7.1.42.I5G

Result	Species ID: Pseudomonas syringae pv maculicola
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.857	0.572	4.809	GN-Nent	Pseudomonas syringae pv maculicola
2	0.061	0.034	6.501	GN-Nent	Pseudomonas resinovorans
3	0.045	0.025	6.692	GN-Nent	Pseudomonas fuscovaginae
4	0.036	0.019	6.843	GN-Nent	Pseudomonas putida

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	0	{ 75	0	0	0	0	0	0	0	< 250	< 250	< 250
B	0	0	0	0	0	0	0	0	0	< 250	75	0
C	0 +	0	0	0	0	0	0	{ 75	0	< 250	< 250	< 250
D	{ 75	{ 75	0	0	0	0	0	0	0	< 250	< 250	< 250
E	0	0	< 250	< 250	{ 75	< 250	< 250	{ 75	0	< 250	< 250	< 250
F	0	< 250	{ 75	{ 75	< 250	{ 75	< 250	< 250	< 250	< 250	< 250	< 250
G	{ 75 +	0	0	< 250	< 250	< 250	{ 75	< 250	0	< 250	0	75 +
H	0	< 250	0	< 250 -	0	0	{ 75	{ 75	< 250 -	75	75	75

Report Date Nov 18 2016 2:30 PM

Fig. 5.12.Detection of the isolate by Biolog Gen III microplate

5.9. 16S rRNA gene isolation and sequencing analysis:

16S rRNA gene sequencing has been established as the gold standard for identification and taxonomic classification of bacterial species. The hyper variable regions of 16S rRNA gene sequences provide species-specific signature sequences useful for bacterial identification. In this work, comparison of the bacterial 16S rRNA sequence has been emerged as a valuable genetic technique and can lead to the recognition of novel bacterial enzymes in *Pseudomonas* species. The sequence of the phenol degrading bacteria *Pseudomonas syringae pv maculicola*is

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGACGGGAGCTT
GCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAG
GAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGG
TCGGATTAGCTAGTTGGTCAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCA

CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGATTATTGGACAATGGGCGAAAGCCTG
ATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAAT
TAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACGGGTTAACTTCGTGCCAGCAGCCGCGGTAATACGA
AGGGTGCAAGCGTTAATCGGAATTACTGGGCGTATAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCC
CGGGCTCAACCTGGGAACTGCATCCAAATCTGGCGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAG
CGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGAAACTGACACTGAGGTG
CGAAAGCGTGGGGAGCAATCAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAA
TCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACG

5.10 Taxonomic identification of isolates:

On the basis of the consensus sequences for the 16S rRNAgene, a phylogenetic tree was constructed using sequences from strain isolates and representative bacteria and the bacteria is mentioned as AUBT-PR. The dendrogram below showing the evolutionary relationship of the *Pseudomonas syringae pv maculicola which is represented as* AUBT-PR. The dendrogram was based on 16S rRNA gene sequence and was constructed by neighbor-joining method

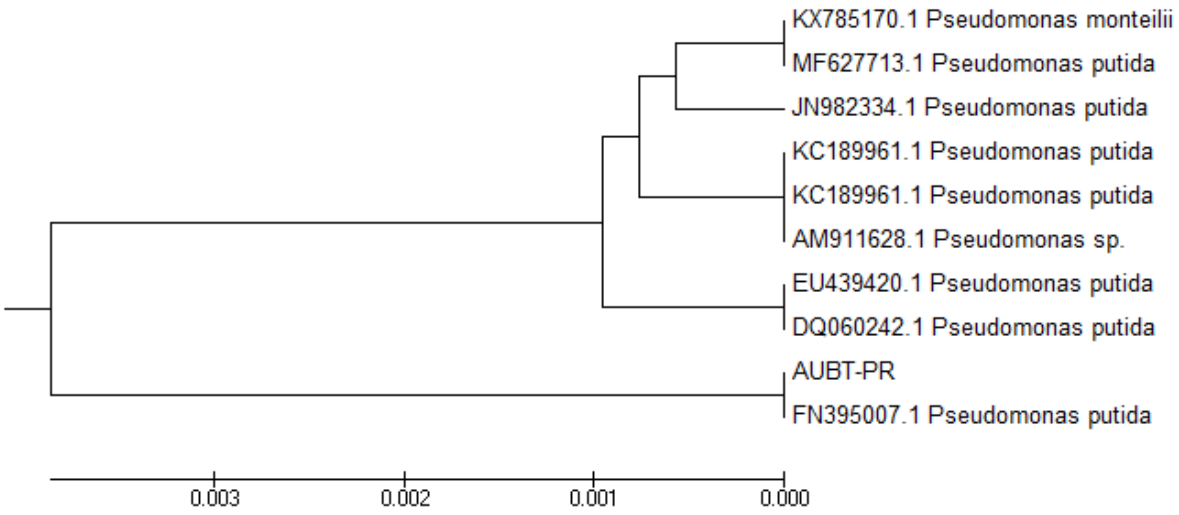

Fig.5.13.Evolutionary relationships of taxa

The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.01001067 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood

method [2] and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 879 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

5.11 PCR Detection of Key Functional Genes in Phenol-degradation Pathways:

To determine whether the catechol enzymes were present, PCR amplifications were performed using catechol1,2-dioxygenase(1,2-CTD),catechol2,3-dioxygenase (2,3-CTD) primers, which are specific for *Pseudomonas syringae pv maculicola.* The expected fragment was amplified from genomic DNA tha was isolated from the respective bacteria.

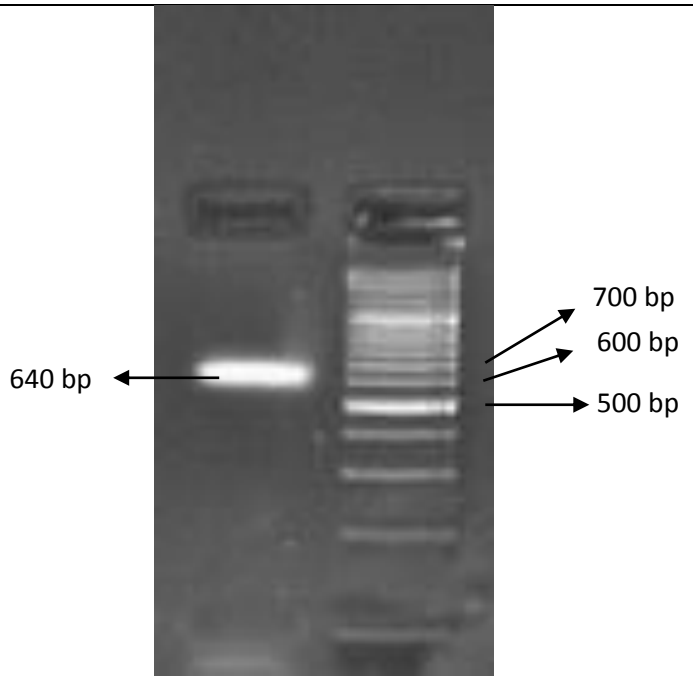

Fig 5.14. Andaromatichydroxylase a-subunits (TBMB) amplified 640bp PCR amplicon in agarose gel

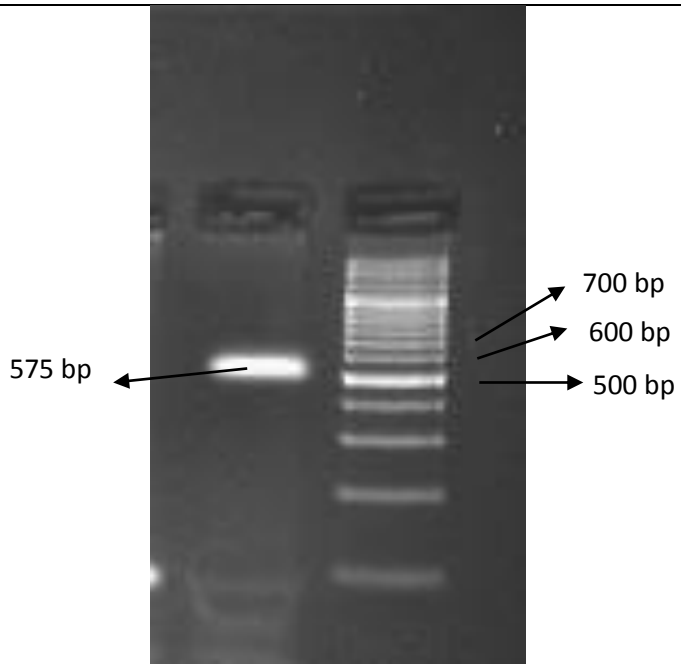

Fig. 5.15. Phenol hydroxylase(Lph) amplified 575bp PCR amplicon in agarose gel

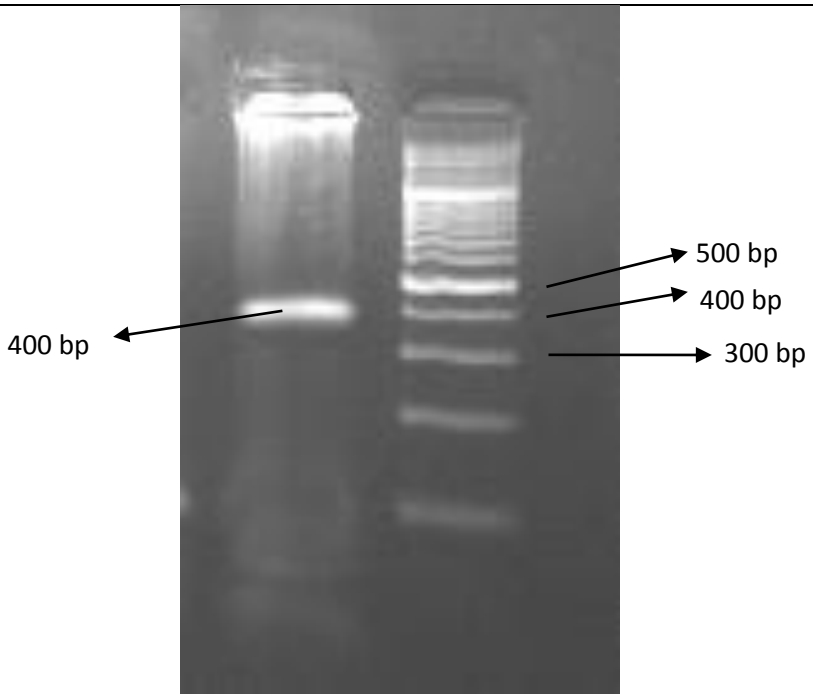

Fig. 5.16. catechol 2,3-dioxygenase (2,3-CTD) amplified 400bp PCR amplicon in agarose gel

The phenol biodegradation pathway reveal that the phenol- enriched culture produce phenol hydroxylase and catechol 2, 3 -dioxygenase, which indicate that the enrichment culture could degrade phenol by the meta-pathway. Meanwhile, many strains degrade phenol via meta-pathways, in which phenol hydroxylase and catechol 2, 3-dioxygenase are induced.

In the ortho- and meta-pathways, catechol ring is decomposed into acetate, succinate, pyruvic acid, and acetaldehyde.

5.12 Determination of Kinetic Parameters:

From this plot, the specific growth rate was increased with an increase in the initial phenol concentration to a certain level and then a decrease was started with a more increase in the concentration. This trend suggested that the phenol is inhibitory substrate. In general, Haldane's growth kinetics model is used to represent growth kinetics data of an inhibitory compound such as phenol. The obtained specific growth rates for the media with low-concentration were also fitted.

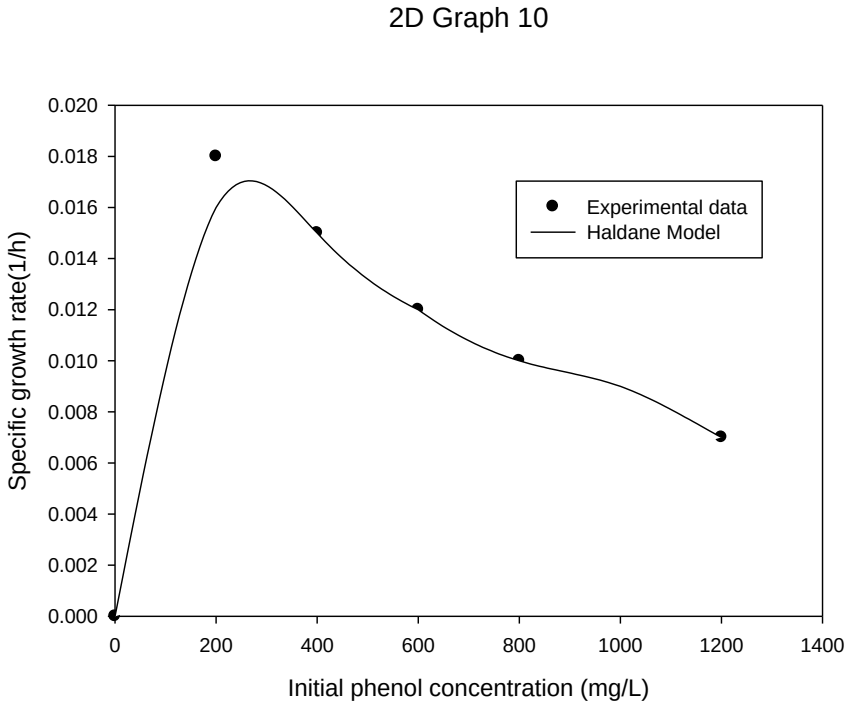

Fig. 5.17 Biokinetics parameter of culture media due to Haldane's growth model

Table 5.12.Comparision of growth kinetics of Haldane Model in various studies for the treatment phenolic wastes

Microbial Strain	Type of Phenolic waste	Concentration range (mg/l)	μ_{max}(h^{-1})	K_s(mg/l)	K_i(mg/l)	Reference citation
Pseudomonas syringae pv maculicola	Phenol	200-1200	0.035	62.3	460	This Study
Pseudomonas putida	Phenol	300-1000	0.031	63.9	450	B. Zeinab et al.,
Mixed culture	Phenol	200-1200	0.003	700	966	R. Neufeld

						et al.,
Mixed culture	Phenol	4000	0.0277	0.03	363	I. Najm et al.,
Mixed culture	Phenol	440-1920	-	-	-	B. Logan et al.,
Pseudomonas putida DSM 548	Phenol	1-100	0.436	6.19	54.1	A. Monteiro et al.,
Pseudomonas putida	Phenol	14-200	0.119	5.27	377	G. Kotturi et al.,
Pseudomonas putida CP1	2,4-dichlorophenol	50-750	1.28	427	1330	F. Kargi et al.,

6. DISCUSSION

In this study, Pseudomonas sp. strain DT-4, capable of degrading phenol, was isolated from industrial effluents. The isolate could use phenol as only carbon source and energy source for growth under incubation conditions. The degradation rate of phenol during the growth phase of strain DT-4 that the experimental and predicted values are 0.0872 and 0.0865 respectively, indicating that the phenol degradation depending on the growth of *Pseudomonas sp*. strain DT-4 by response surface methodology. The isolate grew well in the phenol medium, in which phenol could be degraded almost completely during 96 h of incubation period with stable pH. Studies show that Pseudomonas sp. could degrade 91% of phenol under the optimal growth conditions for 96 h of incubation. Characterization of isolated strain by Gen III Micro log report shows that phenol-degrading bacteria belongs to *Pseudomonas species Pseudomonas syringaepvmaculicola* which had high activity for phenol degradation. However, according to results the characters are closely resemble the *Pseudomonas* species, *Pseudomonas resinovorans*, *Pseudomonas fuscovaginae,Pseudomonas putida.* Conditions optimum for growth and degradation of phenol for *Pseudomonas sp*. strain are intently related. This refers there could exist potential applications for resolving the rise of phenol pollution problem using a biotechnological process via the high capacity and functional gene for degradation of phenol by *Pseudomonas sp.* strain DT-4.The isolate DT-4 was immobilized for effective degradation. The immobilized cells have higher degradation rate constant and wider pH range than that of free cells. The ANOVA of the regression model demonstrates that the model is highly significant Previously, several phenomena had been described during bacterial degradation, and this study reported that isolated strain belongs to Pseudomonas family from the effluents, capable of Phenol metabolization and then continuously degrading after a certain period.

The goodness of the fit of the model was checked by the determination coefficient (R^2). In this case, the value of the determination coefficient isR^2= 0.97247 for free cells and R^2=0.99369 for immobilized cells which indicates there was a satisfactory correlation between the experimental and predicted values of phenol degradation, wherein, the points cluster around the diagonal line indicated the optimal fit of the model, since the deviation between the experimental and predicted values was minimal. The present study have revealed that the more effective degradation of phenol at higher concentration could be

achieved by immobilized cells than freely suspend cells. The immobilized microbial system has an advantage of enhanced rate of degradation, tolerance to higher substrate concentrations and their reusability. Thus, the immobilized microbial technology provides a highly versatile and cost-effective approach that can be used for degradation of phenol contaminated effluents.

Different methods have been used for the elimination of phenol and phenolic compounds, but the use of biodegradation methods is universally preferred because of their lower costs and the possibility of complete mineralization. Bacteria that have the ability to use phenol can be used for biodegradation within environments that are contaminated with phenolic compounds. PCR assays revealed that the three genes were not equally distributed in the isolated strains, and the catechol2,3-dioxygenase gene was found in only *Pseudomonas* sp. The phylogenetic analysis showed that the phenoliccompound-degrading bacteria shared high 16S rDNA gene sequence similarities with one another. PCR analysis was performed to detect dioxygenase genes encoding catechol 2,3-dioxygenase, catechol 1,2-dioxygenase,and protocatechuate 3,4-dioxygenase, which oxidize catecholor protocatechuate via the _-ketoacid and the _-ketoadipatepathways. The identity of the PCR-amplified fragments was further verified through a sequence analysis of selected strains. The three dioxygenase genes were amplified, whichmeans that both the _-ketoacid and _-ketoadipate pathwaysserve as general mechanisms for the catabolism of cate-chol or protocatechuate derived from phenolic compounds. However, the three genes are not equally distributed in the isolated strains. The phenol biodegradation pathway reveal that the phenol- enriched culture produce phenol hydroxylase and catechol 2, 3 -dioxygenase, which indicate that the enrichment culture could degrade phenol by the meta-pathway. Meanwhile, many strains degrade phenol via meta-pathways, in which phenol hydroxylase and catechol 2, 3-dioxygenase are induced.

Growth Kinetics in the present study, Monod equation was unable to describe the growth inhibition of the microorganism at higher substrate concentrations. The phenol exhibited inhibitory behavior on the growth media. In other to have the acclimatization stage for the culture, the organism showed a short lag phase at high substrate concentration, whereas in the low concentrations the lag phase was absent. Specific growth rates obtained for the media with low-concentration and high-concentration phenol were fitted to Haldane's model. The specific growth rate, half-saturation coefficient and inhibition coefficient for Haldane model were 0.035 h-1, 62.3 and 460 mg/l, respectively. Haldane model was successfully applied and perfectly fitted with the experimental data for degradation of phenol.

The results also showed that Pseudomonas syringaepvmaculicolawas able to degrade the phenol at high concentration.